Vol. XXXVIII August, 1949 No. 8

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## *CONTENTS OF THIS ISSUE*

The Biologic Assay of Senna ..... 417
*Lloyd C. Miller* and *Evelyn B. Alexander*
A Pharmacological Study of N-Methyl-N-Cinnamyl-2-Phenyl-Propylamine ..... 425
*Raymond P. Ahlquist, Bertha Hohensach*, and *R. A. Woodbury*
Some Toxicological Properties of Surface-Active Agents ..... 428
*S. S. Hopper, H. R. Hulpieu*, and *Versa V. Cole*
A Summary of the Pharmacological and Toxicological Properties of Anthallan (3-Di(n-butyl)aminomethyl-4,5,6-trihydroxy-phenylphthalide) ..... 433
Edited by *Lloyd W. Hazleton*
Determination of *d*-Tubocurarine Chloride ..... 438
*Daniel Klein* and *Samuel M. Gordon*
A Practical Method for the Biological Assay of Heparin ..... 441
*M. N. Lewis* and *Francesco De Maria*
A Contribution to the Chemistry and Toxicology of the Root of *Phytolacca americana*, L. ..... 443
*Z. F. Ahmed, C. J. Zufall*, and *G. L. Jenkins*
The Muscle Relaxant Effects Produced by *Potentilla anserina* Extracts. I. Fractionation Studies ..... 448
*H. W. Youngken, Jr., A. C. Neva, H. J. Dauben, Jr., Y. W. Chang*, and *E. Wenkert*
The Metabolism and Toxicity of Salicylic Acid in Combination with Various Drugs ..... 451
*Georg Cronheim, Julius Wiland*, and *Michael Ehrlich*
An Unusual Impurity in Caffeine ..... 456
*Harry M. Barnes, Franklin Kramer*, and *Thomas R. Wood*
Viburnum Studies. XVI. Rate of Extraction of Uterine Sedative Potency ..... 457
*Aaron B. Sloane, Albert R. Latven*, and *James C. Munch*
Some New Procedures and Instruments Useful for Microbiological Antibiotic Testing by Diffusion Methods. I. A. New Zone Reader ..... 459
*W. W. Davis, J. M. McGuire*, and *T. V. Parke*
Some New Procedures and Instruments Useful for Microbiological Antibiotic Testing by Diffusion Methods. II. An Automatic Delivery Pipette for Use with Porous Disks ..... 462
*W. W. Davis* and *J. M. McGuire*
Some New Procedures and Instruments Useful for Microbiological Antibiotic Testing by Diffusion Methods. III. The Use of Prepared Tables for Converting Zone Diameters to Potencies ..... 464
*W. W. Davis, J. M. McGuire*, and *T. V. Parke*
Acetamide Derivatives ..... 466
*Charles O. Wilson*
A Proposed Method of Isolating Plant Constituents ..... 468
*George D. Curts* and *Loyd E. Harris*
A Phytochemical Study of *Malva rotundifolio*, L. ..... 470
*George D. Curts* and *Loyd E. Harris*
The Chemical Assay of Aureomycin ..... 473
*Joseph Levine, Edward A. Garlock, Jr.*, and *Henry Fischbach*
Book Reviews ..... 476
Advertising ..... Cover pages 2, 3, 4 and pp. I–IV

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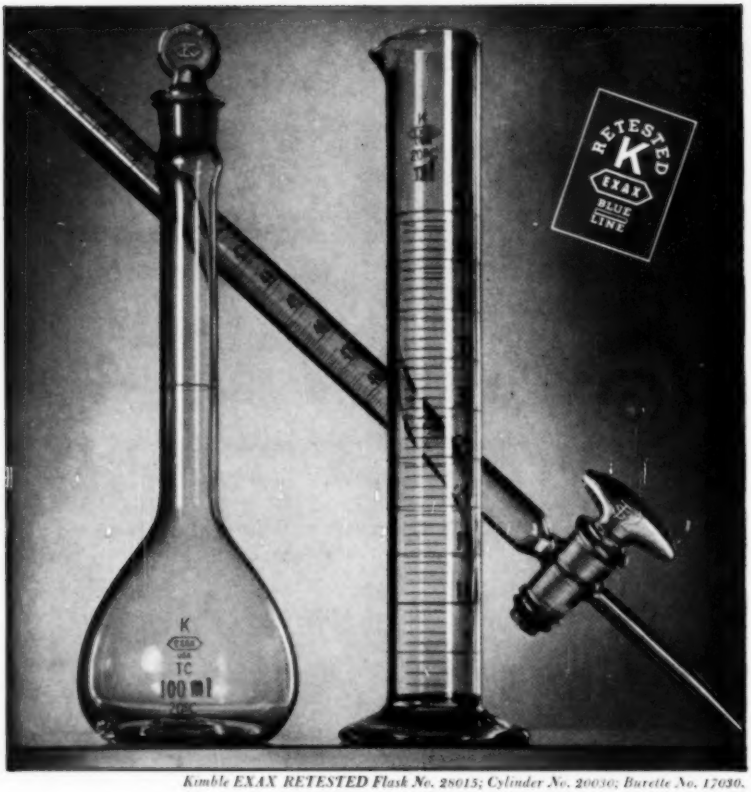


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Annual Subscription—Journal of the American Pharmaceutical Association, complete (both editions): United States and Pan America, $7.00; Canada, $7.70; other foreign, $8.00; members of the American Pharmaceutical Association with dues, $4.00. Each edition, Scientific Edition or Practical Pharmacy Edition: United States and Pan America, $4.00; Canada, $4.35; other foreign, $4.50. Single numbers, either edition: United States and Pan America, $0.35; Canada, $0.40; other foreign, $0.50.

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Entered as second-class matter January 23, 1917, at the Post Office at Easton, Pa., under the act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the 5th; Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

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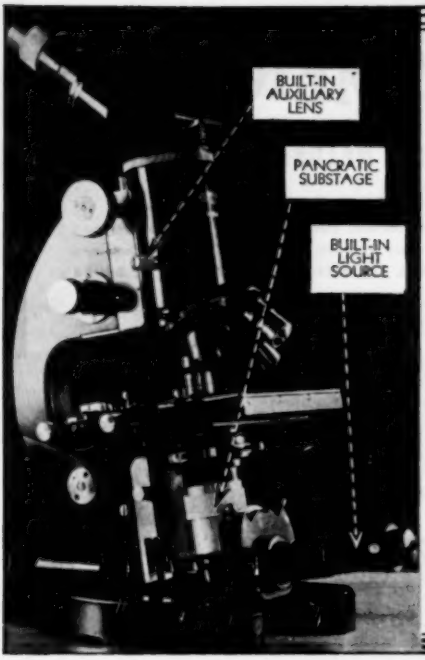


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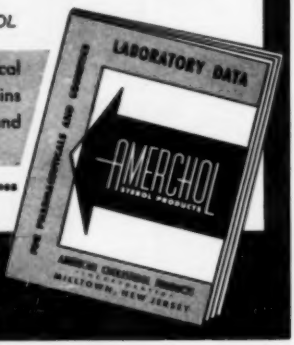


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JUSTIN L. POWERS, EDITOR, WASHINGTON, D. C.

VOLUME XXXVIII — *AUGUST, 1949* — NUMBER 8 — CONSECUTIVE NO. 16

## The Biologic Assay of Senna*

By LLOYD C. MILLER and EVELYN B. ALEXANDER†

**It has been found that rodents are suitable test animals for use in determining the potency of senna, and in the present work mice were used. The assay procedure described involves administering the preparation under test at three dosage levels selected with a view to producing positive laxative effects in approximately 25, 50, and 75 of three similar groups of mice. Concurrently, a suspension of a standard senna powder was given at three appropriate dosage levels. The number of mice on each dose of unknown and standard showing laxative effects during the five and a half hours after dosing were recorded and from these data the potency and confidence limits were calculated by conventional methods. The method is applicable to senna in its various pharmaceutical forms. Satisfactory dosage-response relationships were found in experiments on humans. The potency ratios found on human subjects agree remarkably well with those on mice. This finding appears to validate the biological assay on mice and to facilitate stability studies on senna and its preparations.**

ALTHOUGH SENNA was introduced into medicine in the ninth century (1), no serious attempt has yet been made to set up potency standards for any of the forms commonly seen in pharmaceutical commerce. This situation can be attributed only to a lack of an assay method since hundreds of tons of senna are imported annually. However, in spite of much study, no specific chemical reaction has been found for its active principles and a biologic assay has not been considered seriously until recently. Indeed, not until 1925 were the effects of senna on laboratory animals described even in qualitative terms (2, 3); it remained for Straub and Gebhardt (4) to show in 1936 that the reaction in mice was quantitative enough for assay purposes. Geiger also used mice for assay purposes in 1940 (5). Hazleton and his associates (6, 7, 8) reported observations on mice given senna incidental to other studies, while Grote and Woods (9) advanced the assay substantially to its present state of development by introducing a reference standard senna powder. Wiebelhaus and Lee (10) used young adult rats in determining the activity of fluidextracts of senna. Vichover (11) has advocated the use of *Daphnia magna* for the

* Received August 14, 1948, from the Biology Division, Sterling-Winthrop Research Institute, Rensselaer, N. Y.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August 11, 1948.
† The authors are deeply indebted to Dr. J. O. Hoppe for material assistance in the latter part of this study, to Mrs. Mary Lenz and Mr. Calvin Zippin for aid in the computations, and to Mrs. Nettie Beglin for help in some of the assays on mice.

assay of several cathartics, including senna, and Green, King, and Beal (12) used both guinea pigs and rats in assaying cascara, the former species being preferred.

This study arose out of the necessity for determining the potency of senna preparations in connection with developmental work and stability tests. The material under study consisted of experimental and commercial batches of proprietary preparations containing about 50 per cent sugar and senna extractives equivalent to 50 to 100 mg. of fresh drug per cc.

When the investigation began, the publication by Grote and Woods (9) had not appeared and a review of the available literature indicated that rats might offer some advantages over mice. As the work progressed the converse proved true; further, certain improvements in the use of mice were effected by applying principles of experimental design and analysis which have recently been shown to aid materially in biological assay. Finally, there remained the question of whether the relative potency revealed by the mouse potency assay was a true reflection of the efficacy in humans. It is the purpose of this report to propose a simple, practical assay technique and to demonstrate that the results obtained with it agree remarkably well with those obtained on human subjects.

## EXPERIMENTAL

**Observations on Rats.**—In the preliminary work directed toward developing a biologic assay using rats, it was found that senna readily produced a characteristic catharsis about three hours after dosing. At this time the feces changed in nature from the normal, relatively solid pellets to a formless semi-fluid collagenous mass; in the course of the next three to five hours there was a return to normal. On the theory that under the influence of the senna there was little, if any, resorption of water in the colon, it seemed possible that the weight of the feces or some value derived therefrom might be correlated sufficiently well with senna dosage to be made the basis of an assay. This possibility was explored and the technique finally evolved consisted of distributing pairs of young adult rats to small cages. The floors of the cages were of hardware cloth, two wires to the inch, and were raised to permit collection of the feces at hourly intervals. The rats were allowed food and water during the test.

As indicated above, the test materials had a high sugar content and it seemed desirable to provide that regardless of the senna content, each rat would receive practically the same amount of sugar and fluid. This was accomplished by diluting all doses to the same volume using a senna-free diluent similar to the vehicle of the test preparations. The control rats received a "full dose" of the diluent alone. The volume was generally held at 1.0 cc. per rat given by stomach tube to insure uniformity of dosing both from the standpoints of quantity and time. A fifteen-minute period was allowed for the rats to recover from the stimulation of handling following which glazed cardboard was placed beneath the cages to facilitate collection of the feces. At hourly intervals thereafter the feces were transferred to small beakers tared to the same initial weight with mineral oil; the latter simplified taking the weights and prevented loss of water from the feces during the collection period. Weighings made each hour gave data on the hourly excretion and the cumulated totals.

The doses of the senna syrup ranged from 0.12 to 1.0 cc. per rat. An analysis of data obtained in six- to nine-hour periods showed that during the first three hours the senna-dosed rats excreted no greater weight of feces than the controls. During the next three hours there was an increase attributable to the senna but seldom was there any relation between the amount of drug and the fecal weight. In some trials, the observations were continued through the ninth hour after dosing when the effect was practically over; these added data did not change the picture materially. The conclusion was reached that the shortcomings of rats in assaying cascara observed by Green, King, and Beal (12) appear to hold also for senna. Work on rats was therefore discontinued in favor of studies on mice.

**Observations on Mice.**—Geiger's procedure (5) as modified by Grote and Woods (9) was followed in using adult male Webster mice kept on a diet of commercial laboratory feed in checker form (Rockland Farms brand). Care was taken to insure that ample food and water were available during the twenty-four hours prior to the test. On the test day the mice were transferred to small cages, the floors of which were constructed to permit the fecal excreta to drop freely onto clean, absorbent paper placed 1 to 2 cm. beneath the cages. For convenience, the cages were constructed in sets of four with solid sides and hinged covers; experience showed that hardware cloth having three small-gauge wires to the inch was best for the floors.

The mice were dosed by stomach tube, using a $1^1/_2$-inch, 18-gauge hypodermic needle blunted on the end with a tear-drop of solder. To facilitate their administration, senna syrups were diluted with water while powders (reduced to 80 mesh fineness) were suspended by treatment with boiling water, 20 cc. per Gm. of powder; the resultant suspensions were shaken occasionally while cooling in air for about thirty minutes. The stock solution or suspension was diluted further with water so that each dose selected was contained in 0.5 cc. As suggested by Grote and Woods, a senna powder was set up as a provisional reference standard and each test preparation was compared with suspensions of it. Handling suspensions was not wholly satisfactory but was followed as part of the method of Grote and Woods; results with supernatant fluid obtained from centrifuged suspensions indicated that these are fully active or at least representative of the powders used.

**Dosage-Response Relationship.**—In preliminary work it was quickly found, in confirmation of earlier reports (4, 5, 9), that mice discriminate between graded doses of senna preparations with reasonable consistency. To determine the character and stability of the dosage-response relationship, 5 groups of 22 previously unused adult Webster mice were set

aside. The groups were assigned doses in accordance with a Latin square design which provided that each group received a different dose each test day and no group received the same dose twice. Five dose levels in a geometric series were obtained by diluting a freshly made suspension of 2.5 Gm. of standard senna powder in 50 cc. of water so that 0.5-cc. portions contained 4.0, 5.6, 8.0, 11.3, or 16.0 mg. of senna powder. Following preliminary observation the mice were dosed by stomach tube and returned to their cages in a room held at a temperature of 23 to 25°. Observations were made at five hours and each mouse was identified so that an individual record of its responses was kept.

The observations were quite elementary and consisted in noting whether or not any feces had been passed which were fluid enough to cause a surrounding ring of imbibed stain to appear in the paper. Previous workers (9) have suggested examining the reverse side of the paper as a means of distinguishing stains produced by drops of urine. This proved inconvenient and unnecessary.

The data are given in Table I. It is apparent at once that a slightly higher range of doses might well have been used; the over-all response was only 32.5%. However, the almost complete lack of response to the 4-mg. dose shows that the process of dosing must be virtually without effect *per se*. Hence for practical purposes, the group on this dose each day served as a "control."

With respect to the reactions of the individual mice, a study of the results reveals that three mice showed no positive effects after any dose. Against this manifestation of low sensitivity there was no evidence of high sensitivity in that no mouse responded to all five doses; however, one mouse responded to all of the four higher doses. Three mice reacted to the two low doses but not to any of the three higher doses; finally, 15 mice responded only to the three higher doses. From these data it appeared that the reaction was typically quantal ("all-or-none") in nature, so that plotting the percentage response against dose should yield an asymmetric sigmoid curve which could be rectified by the probit-log dose transformation. That the latter is true is seen in Fig. 1 which gives the results obtained on the five test days, each plotted separately but to the same scale. In plotting the data the convention suggested by Miller and Tainter (13) was followed for the cases of zero response.

The dosage-response curves for each test day were calculated by the method of Bliss (14) and the computed lines of best fit are drawn in through the plotted points of the observed data. The parameters of the curves are given in the lower part of Table I. The chi-square test for linearity (14) indicates that straight lines fit the plotted points within the limits of normal variation. The slope, or steepness of the lines, is fairly constant from day to day, although by chance, that observed on the second day was almost significantly greater than that of the first

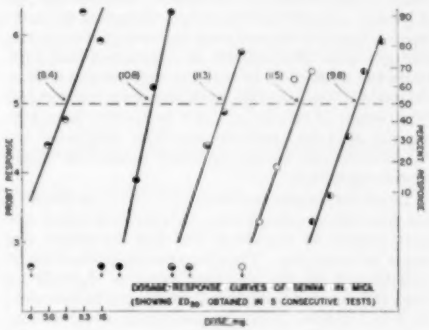


Figure 1

test. For the series of 5 tests the variation in slope was not significant ($\chi^2 = 5.57$, $P = 0.24$); however, it was sufficiently great to suggest giving each preparation at two or more dose levels to provide an estimate of the slope for each assay. The $ED_{50}$, or the quantity estimated to be necessary for producing positive responses in 50% of a group, did vary significantly from day to day; this may be judged from the standard errors given in the last line of Table I. The $\chi^2$ value for the series was 12.45, indicating that the probability was only one in fifty ($P = 0.02$) that such heterogeneity would be encountered through normal variation. Obviously

TABLE I.—DOSAGE-RESPONSE RELATIONSHIP FOR SENNA GIVEN BY MOUTH TO MICE

The distribution of positive responses in groups of 22 mice given the doses indicated and the calculated parameters of the dose-response curves

| Dose, Mg./Mouse | Test Day 1 | 2 | 3 | 4 | 5 | Totals for 5 days |
|---|---|---|---|---|---|---|
| 4.0 | 0 | 0 | 0 | 0 | 1 | 1 |
| 5.6 | 6 | 0 | 0 | 1 | 2 | 9 |
| 8.0 | 9 | 3 | 6 | 4 | 7 | 29 |
| 11.3 | 20 | 13 | 10 | 14 | 15 | 72 |
| 16.0 | 18 | 20 | 17 | 15 | 18 | 88 |
| Day Totals | 53 | 36 | 33 | 34 | 43 | 199 |
| Test for linearity $\chi^2$ | 4.21 | 0.18 | 2.40 | 3.35 | 0.90 | |
| Test for linearity $P$[a] | 0.12 | 0.90 | 0.30 | 0.18 | 0.60 | |
| Slope ± s. d.[b] | 4.30 | 8.29 | 4.86 | 4.90 | 4.70 | |
| Slope ± s. d.[b] | 0.74 | 1.54 | 1.19 | 0.99 | 0.76 | |
| $ED_{50}$ ± s. d.[b] | 8.4 | 10.8 | 11.3 | 11.5 | 9.8 | |
| $ED_{50}$ ± s. d.[b] | 0.6 | 0.5 | 0.9 | 0.9 | 0.7 | |

[a] The probability that a straight line adequately fits the points of a log-probit graph.
[b] Standard deviation.

the lower $ED_{50}$ value on the first day (due mainly to a notably high response to the 11.3-mg. dose) was the principal source of this heterogeneity.

**Design of the Assay Procedure.**—From the above-described study of the dose-response data it was clear that senna has much in common with other drugs which give all-or-none responses, such as digitalis on frogs, estrone on spayed rats, etc. Experience with these other drugs indicated three requirements for the biological assay of senna. These are: (*a*) a reference standard drug must be run in parallel with the sample under assay to minimize the influence of variations in sensitivity of the mice; (*b*) the standard and sample must be run at two or more dosage levels to provide an estimate of the slope and thereby the basis for estimating the confidence limits of the potency estimate; and (*c*) the multiple dose levels should be selected so that each bears the same ratio to the next highest and so that the ratio is the same for both standard and sample. Observance of this latter point facilitates interpreting the data by available simplified methods; for the same reason, an equal number of animals should receive each dose.

There is a serious conflict of objectives in choosing the number of dose levels. A two-level assay unquestionably is simpler to run and its results are easier to calculate. Thus for routine work, a slight modification of the two-dose assay of digitalis on frogs (15) proved fairly satisfactory and is described in detail below. Certain provisions were written into this as safeguards against the anomalous results encountered occasionally, such as zero or 100% response, or less frequently, a greater response to the low than the high dose. For reasons that were never discovered, these "aberrancies" occurred about twice as often as was to be expected through chance variation. Thus for critical assays, it was found best to use a three-dose procedure such as worked out for the one-hour assay of digitalis on frogs (16). This assay design does not eliminate the troublesome variation, but more clearly shows the extent of the departure from normal expectation and facilitates salvaging useful information from the data.

**Calculation of the Potency and Confidence Limits.**—The choice of the assay design determines how simple may be the potency calculation. The data are quantal in nature, consisting of the proportion of all-or-none responses in groups of mice to either the reference standard or the sample on assay. Two fairly simple procedures have been worked out for the results of the two-level assay (15, 17); the first of these involves the probit transformation and is a simplification of the maximum likelihood solution, while the second calls for angular transformation. Both methods give substantially the same result but the probit method has been chosen on theoretical grounds.

The three-level data are less readily calculated. The standard method is the maximum likelihood solution (16) but this is somewhat tedious even if carried out on an electric computing machine; for the purpose of this assay, it suffices to round off the derived data to the point where a slide rule can be used. DeBeer (18) and Litchfield and Wilcoxon (19) have published combination graphic-nomographic methods for the maximum likelihood solution. The Litchfield-Wilcoxon method does not involve transformation of the original data and avoids weighting by assuming an average weight for all points; it appears to be the method of choice for three-level data.

**The Reference Standard and Its Use.**—As stated above, Grote and Woods (9) introduced the use of a reference standard senna powder which consisted of "exceptionally bright green Grade 1 Tinnevelly senna...ground to pass an 80-mesh sieve and packed carefully into small tightly sealed bottles." Through the kindness of Dr. Grote, some of this standard was made available for use in most of the early work of this study. Later, a secondary standard was prepared and standardized in terms of the original powder. The dry powder appears to be entirely stable at room temperature.

Grote and Woods (9) administered the standard senna to the mice as a suspension in water. This practice was followed in the experiment, yielding the data given in Table I above. However, the suspension settles quickly and tends to jam syringes. It has been found that the fairly clear supernatant fluid obtained by centrifuging is equal in potency to the whole suspension from which it was prepared. Thus the use of the supernatant has been specified in the directions below.

### The Assay Procedure

**The Standard Preparation of Senna.**—Weigh accurately about 2 Gm. of standard senna powder and transfer it to a glass-stoppered graduated cylinder. Add 50 cc. of boiling water and allow the mixture to cool to room temperature with occasional shaking. When cool, or about thirty minutes later, centrifuge and decant the supernatant liquid for use as the standard preparation. If kept under refrigeration to protect against fermentation, this preparation may be used as long as a week.

**The Mice.**—Select mice weighing between 20 and 30 Gm. which are free of gross evidence of disease or injury and which have had free access to food and water during the previous twenty-four hours. Distribute the mice into individual cages which are constructed to permit fecal excretions to drop freely onto white filter paper placed 1 to 2 cm. beneath the cages. Observe the mice for about thirty minutes and discard any which are excreting feces fluid enough to stain the absorbent paper.

**Preparation of the Test Dilutions.**—Select, by preliminary trial, if necessary, two doses of both the standard and the sample on assay such that the low dose, when given to each of sixteen mice, will be expected to produce positive reactions in about five mice and the high dose, when given similarly, will be expected to produce positive reactions in about eleven mice. Select these doses so that the ratio of the high dose to the low dose shall be the same and not less than 1.5 nor more than 3.0 for both standard and the sample on assay. Dilute the standard preparation of senna and the preparation being assayed with water, so that 0.5 cc. contains the dose for each mouse.

**Administration of the Test Dilutions.**—Following the initial observation period of thirty minutes, administer 0.5 cc. of the respective test dilutions by means of a syringe fitted with a suitable blunt-tipped tube which is approximately 1 mm. outside diameter and 40 mm. long (a 1½-inch, 18-gauge hypodermic needle). Return each mouse to its

cage and at the end of five hours examine the paper beneath the cage. Consider that a mouse showed a "positive" reaction during the observation period if its feces stained the paper.

**Calculation of the Potency.**—From the proportion of each group of mice showing positive reactions on each test dilution calculate the potency by the procedure described for the two-dose, eighteen-hour digitalis assay on frogs (15).

A sample calculation of a typical assay is given in Table II. This example shows all the steps of the calculation without, however, giving every intermediate step. The latter are shown clearly in the original publication (15). Although, as given, the calculation requires only simple addition and subtraction abetted by a good slide rule and a table of squares, it may be simplified greatly by preparing tables for the values of $W$, $\bar{x}$, and $\bar{y}$ corresponding to all reasonably possible combinations of the basic data.[1] The limiting value of 0.30 given in Table II of 20% can be discriminated. It has been noted that infrequently, but more often than would be expected in chance variation, a clearly aberrant result is obtained. Usually this is manifested in a greater response to the low dose than to the high dose and it is scarcely attributable to personal error. Assuming the latter is true, the safeguards written into the calculation procedure, "the Chi-Square Test for Validity" and "Determination of the Average Sensitivity to a Difference in Dosage" (15) are adequate for indicating when assays showing aberrancies of this kind should be repeated.

**Parallel Assays of Senna Preparations on Mice and on Human Subjects.**—Several samples differing significantly in potency were encountered. Generally, these were known to differ in age, method of manufacture, or actual senna content. There remained, however, the question of whether these differences were real from the standpoint of efficacy in man. To investigate this point, three assays were conducted on volunteers from the laboratory staff. It proved possible to balance out much of the influence of the immense individual variation by an experimental design, particularly in the second and third assays. Two or more dose levels of each test preparation were given according to a schedule whereby, at weekly intervals, each subject received a different dose. The order of dosing was determined in advance by a Latin square; to reduce subjective bias, all doses were made up to the volume of the largest with either water or flavored inert syrup.

Report forms were submitted daily for three days prior to the test days to establish the pattern of the subject's bowel habits; any indication of irregularity thereby revealed was cause for postponing a test on that subject. The test dose was swallowed, with very few exceptions, in the presence of one of the authors; its identity was not revealed to the subject who was asked to record the time and character of his evacuations during the next twenty-four hours.

In the first assay, it was of interest to compare five samples which varied widely in potency in mice. The mouse assays on these samples were carried out early in the study before certain refinements had

TABLE II.—NUMERICAL EXAMPLE OF THE STEPS IN CALCULATING THE POTENCY OF A TWO-DOSE ASSAY OF SENNA

| | Basic Data | | | Derived Data | | | | |
|---|---|---|---|---|---|---|---|---|
| Prepn. | Dosage | Log Dose | Observed Response | $Y$ | $w$ | $wY$ | | |
| Standard | | | | | | | | |
| Dose 1 | 8 mg. | 0.903 | 2/16 | 4.11 | 4.2 | 14.57 | $W_s$ = | 2.89 |
| Dose 2 | 16 | 1.204 | 11/16 | 5.49 | 9.3 | 51.06 | $\bar{x}_s$ = | 1.029 |
| | | | | Sums | 13.5 | 65.63 | | |
| | | | | Diff. 1.38 | | | $\bar{y}_S$ = | 4.86 |
| Unknown | | | | | | | | |
| Dose 1 | 0.16 cc.[a] | 0.796 | 4/16 | 4.33 | 8.6 | 37.24 | $W_u$ = | 2.82 |
| Dose 2 | 0.32 | −0.495 | 15/16 | 6.53 | 4.2 | 27.43 | $\bar{x}_u$ = | −0.697 |
| | | | | Sums | 12.8 | 64.67 | | |
| | | | | Diff. 2.20 | | | $\bar{y}_u$ = | 5.14 |

$\chi^2 = 0.96$ ($< 2.7$) so that data on standard and unknown are consistent.
$s_e = 0.169$ ($< 0.3$) so that mice were sufficiently sensitive to differences in dosage.
$M = 1.7732$; antilog $M = 59.3$ mg. senna per cc.
$s_M = \pm 0.066$; standard error = $\pm 9.0$ mg. senna per cc.

[a] By multiplying the dosage by 10, negative logarithms are avoided; appropriate correction must be made later by adding 1.00 to $M$.

for $s_e$ has been arrived at from data obtained in this study. It is a measure of the capacity of the mice used to discriminate differences in dosage of senna. The suggested value may not be entirely appropriate for universal use, although it is unlikely that marked differences in discriminatory power due to strain variation, etc., will be encountered.

### Assay Observations

**Error of the Mouse Assay.**—A number of factors contribute to the error of this assay; the inherent heterogeneity of the mice is probably the greatest source of variation as manifested in the individual responses discussed above. This heterogeneity could be reduced somewhat by culling out the mice showing the extremes of sensitivity to senna. However, in 57 consecutive routine assays using 16 unselected mice on two doses of both standard and sample, the standard error averaged $\pm 21\%$ of the observed potency. The values of individual assays ranged from 13% to 43%. This suggests that by replication, differences in potency

[1] Copies of the tables for assays on 16 mice per dose level are available upon request from the authors.

been adopted; as a result, the errors are larger than those of later assays. However, the potency differences were clearly significant. Four of the samples were proprietary senna syrups, comparable in composition; Samples A, D, and E were of recent manufacture while B was eight years old. Sample E differed from A, B, and D in having been made in percolator of improved design, so that more efficient extraction was obtained from the same quantity of senna leaves. Sample C was prepared by adding fresh senna concentrate to a flavored syrup to simulate the other samples.

With so many samples it was impractical to carry out a completely balanced experiment whereby each of the 40 subjects would receive both a low and a high dose of every sample. The low doses of all five samples were given to one-half of the subjects and the high doses to the rest; while this design favored the comparison of the various preparations at a given dose level, it did not permit a good estimate of how well differences in dose could be discriminated. Unfortunately also, not all of the subjects in each group completed the experiment and this still further complicated the comparisons.

In both the later assays, only one preparation was compared with another taken as the standard so that a much simpler experimental design was possible. In the second assay, a senna syrup prepared in the laboratory by a slightly modified formula was compared with the commercial preparation of the original formula. The object of the third assay was to compare two commercial syrups made by the same process but differing in age; both were pooled specimens of eight lots manufactured within a thirty-day period. The lots making up Sample H were manufactured in January, 1948, while Sample I consisted of the reserve samples of lots manufactured almost exactly four years earlier. The latter had been kept at Rensselaer, N. Y., and had been subjected to the seasonal variations in room temperature, etc., during the intervening four years.

The data on Samples H and I illustrate best the potentialities of the assays on both mice and human subjects. The data on mice are given in Table III; this assay was carried out on two days using a total of 24 mice per dose. Sample H was given at four dose levels and Sample I at only three levels, inasmuch as 0.5 cc., the maximum volume that can be given mice without difficulty, gave only a 67% response. Sample I was 35 ± 5% of Sample H, indicating a very significant difference in potency and, assuming that the lots comprising Sample I had originally been substantially the same strength as those of Sample H, the difference was attributable to deterioration.

In comparing Sample I with Sample H on volunteers, 28 subjects were used. Each took six doses representing three levels each of the two preparations. The precautions mentioned above were observed, i.e., the order of dosing was predetermined and balanced, the volume of the dose was kept constant and every effort was made to minimize subjective bias. The data obtained are given in Table IV from which several interesting observations are possible. It is clear that there is a very great difference in the over-all responses to the two samples; indeed, to 12 of the 28 subjects, Sample I was ostensibly inert. In contrast, Sample H produced some effect in every subject. The subjects varied considerably in their inherent sensitivity to the laxative, Subject 9 being the least sensitive and Subjects 3, 15, and 25 appearing somewhat more sensitive than most of the other subjects. The subjective observations of "gas pains" and "griping" are scarcely reliable as criteria of laxative effect.

It appeared however, that the best index to potency was simply the number of "extra stools," i.e., the difference between the number reported in the twenty-four hours following the test dose and the number (or, more exactly, the mode) reported on the three preceding days. That the number of extra stools correlates well with the logarithm of the dose is seen in Fig. 2, which portrays graphically the composite data of Table IV. It is clear beyond question that straight lines fit the respective sets of points and that the two lines are parallel within practical limits. It was thus possible to calculate the potency and its standard error by the method developed by Bliss and Marks (20) for the insulin assay. The result, 34 ± 7%, agrees almost incredibly well with the

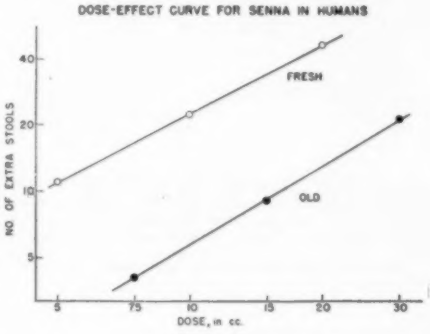


Figure 2

TABLE III.—SUMMARY OF MOUSE ASSAY DATA ON SAMPLES H AND I
24 Mice Used on Each Dose

| | Sample H | | | | Sample I | | |
|---|---|---|---|---|---|---|---|
| Dose, cc./mouse | 0.8 | 0.13 | 0.2 | 0.32 | 0.2 | 0.32 | 0.5 |
| No. positives | 3 | 14 | 21 | 22 | 1 | 13 | 16 |
| Approx. % positives | 12 | 58 | 88 | 92 | 4 | 54 | 67 |

Calculated Potency Ratio: Sample I is 34.6 ± 4.6% of Sample H.

above-mentioned value of 35 ± 5% obtained on mice.

It seems safe to conclude that the good agreement between species obtained with Samples H and I was not entirely fortuitous in view of the data obtained earlier and also listed in Table V. The standard errors of the potency estimates on volunteers in the tive ranks of the five samples, but also there was no significant discrepancy between the corresponding pairs of potency estimates. In the last column of Table V the ratios of the respective mouse and human potency results are given. The greatest discrepancy from unity is seen with Sample B which appeared slightly stronger on man than on the

TABLE IV.—COMPILATION OF INDIVIDUAL RESPONSES OF 28 HUMAN SUBJECTS GIVEN GRADED DOSES OF TWO SENNA PRODUCTS

| Subj. No. | Sample H | | | Sample I | | |
|---|---|---|---|---|---|---|
| | 5 Cc. | 10 Cc. | 20 Cc. | 7.5 Cc. | 15 Cc. | 30 Cc. |
| 1 | 0[a] | 1[b] | 3[b] | 0 | 0 | 0 |
| 2 | 0 | 0[b] | 2[c] | 0 | 0 | 0 |
| 3 | 1 | 1 | 3[b] | 0 | 1 | 1 |
| 4 | 0 | 1 | 1 | 0 | 0 | 0 |
| 5 | 0 | 1[a] | 0 | 0 | 1[a] | 1 |
| 6 | 1 | 1 | 2 | 0 | 0 | 0 |
| 7 | 0 | 0 | 1[a] | 0 | 0 | 0 |
| 8 | 0[a] | 1[b] | 2[b] | 0 | 0 | 1[b] |
| 9 | 0 | 0[c] | 0[b] | 0 | 0 | 0 |
| 10 | 0 | 0 | 2[b] | 0 | 0 | 1 |
| 11 | 0 | 1 | 2 | 0 | 0 | 0 |
| 12 | 1 | 0 | 2[a] | 0 | 0 | 1 |
| 13 | 0 | 3 | 1[a] | 0 | 0 | 0 |
| 14 | 1 | 0[b] | 3[b] | 0 | 2[b] | 1 |
| 15 | 0 | 2 | 2 | 1 | 1 | 4 |
| 16 | 2[b] | 0 | 2[b] | 0 | 2 | 2 |
| 17 | 0[a] | 1[c] | 2 | 0 | 0 | 1[c] |
| 18 | 0 | 0 | 3[a] | 1 | 1 | 0 |
| 19 | 1 | 3[a] | 1[a] | 0 | 0 | 2[a] |
| 20 | 0 | 1 | 3[b] | 0[a] | 0[a] | 2 |
| 21 | 0 | 0[b] | 1[b] | 0 | 0 | 0 |
| 22 | 0 | 0 | 2[b] | 0 | 0 | 1 |
| 23 | 1[b] | 3[b] | 1 | 0 | 0 | 0 |
| 24 | 0[a] | 0 | 0[b] | 0 | 0[a] | 0 |
| 25 | 1 | 1 | 1 | 1 | 1 | 3 |
| 26 | 0[a] | 2 | 0 | 1[a] | 0[a] | 0[a] |
| 27 | 0 | 0 | 3 | 0 | 0 | 0 |
| 28 | 2 | 0[a] | 1[b] | 0 | 0 | 0 |
| Totals: | | | | | | |
| Gas pains | 4 | 5 | 6 | 2 | 4 | 3 |
| Griping | 3 | 7 | 13 | 0 | 1 | 2 |
| Extra stools | 11 | 23 | 46 | 4 | 9 | 21 |

[a] Reported gas pains. [b] Reported griping. [c] Reported both gas pains and griping.
Calculated Potency Ratio: Sample I is 34.0 ± 7.4% of Sample H.

TABLE V.—COMPARATIVE DATA ON SENNA ASSAYS

Preparations Assayed on Both Mice and Human Volunteers

| Assay | Sample | Type of Preparation | Potency on | | Ratio Mouse:Man |
|---|---|---|---|---|---|
| | | | Mice | Men | |
| 1 | A | Fresh Commercial Syrup | 100 | 100 | ... |
| | B | 8-Year-old Commercial Syrup | 52 ± 10 | 63 | 0.83 |
| | C | Fortified Laboratory Syrup | 483 ± 62 | 415 | 1.16 |
| | D | Fresh Commercial Syrup | 149 ± 11 | 154 | 0.97 |
| | E | Fresh Commercial Syrup | 233 ± 28 | 208 | 1.12 |
| 2 | F | Commercial Syrup | 100 | 100 | ... |
| | G | Laboratory Syrup | 79.5 ± 4.4 | 80.9 ± 11.5 | 0.98 |
| 3 | H | Fresh Commercial Syrup | 100 | 100 | ... |
| | I | 4-Year-old Commercial Syrup | 34.6 ± 4.6 | 34.0 ± 7.4 | 1.02 |

first assay could not be calculated by simple means but undoubtedly they were much higher than those actually calculated in Assays 2 and 3. Nevertheless, the differences in the potencies of the samples compared in Assay 1 were sufficiently great to insure clear discrimination if such were reasonably possible. Not only did the two sets of results agree in the relamouse; the difference, however, was far from significant on the basis of the data in hand. Sample C presented a similar situation except that the value observed on the mouse was the greater.

Little need be said of the data of Assay 2 in which essentially identical results were obtained in both species. While the calculated error of the mouse

assay was the lowest observed in the entire series both absolutely and relatively, that for the result on volunteers was nearly three times as large. The latter assay involved two dosage levels, 12.5 and 25 cc., in a balanced experiment on 61 subjects who completed the scheduled series of four doses each. Use of the greater number of subjects yielded a relatively lower standard error than obtained in Assay 3, although in the latter each preparation was tested at three-dose levels as indicated in Table IV. However, in both cases the mouse:human ratio was practically 1.0.

## DISCUSSION AND SUMMARY

Aside from the rather gratifying aspects of these observations from the standpoint of biological assay, it is perhaps not too much to hope that they may serve in several important respects. First, the validity of the assay procedure on mice has been established to a degree of finality seldom achieved. Few drugs can be given with impunity to normal subjects to produce their normal action in such clear-cut fashion. Second, the value of good experimental design is amply demonstrated. The responses of human subjects to cathartics has long been considered vagarious. Although the subjects of these experiments exhibited the normal variability in response, this element was sufficiently controlled by the experimental design to bring into bold relief the differences between samples. Third, it should now be possible to set up potency specifications for senna as for other vegetable drugs and fix the value according to the assayed strength. It follows that experiments may now be conducted with confidence in the mouse potency assay data to indicate the preferred methods of harvesting, drying, and storing the leaves. A fourth point is that stability tests may be made of the various pharmaceutical forms of senna to determine which are the most stable and how greater stability may be attained. Finally, it may now be possible to provide a potency assay in the official compendia for such forms as are adopted therein. Senna leaves and senna fluidextract and syrup are items of U. S. P. XIII; no potency assay is provided for any of these forms. If a biological assay as described here is adopted, it would be necessary to provide a suitable senna reference standard.

While the emphasis of this communication has been on the biological assay, mention should be made of the eminent desirability of developing a chemical assay. Possibly the availability of a refined biological assay method will stimulate a search for a satisfactory correlation between results thereby obtained with some measurement more easily made.

Between the time this manuscript was presented orally and submitted for publication, a communication appeared giving a comparison of a biological and a chemical assay (21). The former differs considerably from that described herein and would appear to be less practical. It is of interest, however, that a fairly good correlation was observed between the results obtained in parallel with the biological and chemical methods of assay.

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## SEVENTH INTERNATIONAL BOTANICAL CONGRESS

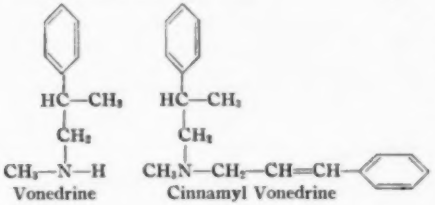
The Seventh International Botanical Congress will be held in Stockholm, Sweden, between July 12 and 20, 1950. The Organizing Committee for the congress requests that all those expecting to attend file a preliminary application as promptly as possible. Complete information relating to plans for the 1950 congress may be obtained from the General Secretary, Ewert Åberg, Uppsala 7, Sweden.

# A Pharmacological Study of N-Methyl-N-Cinnamyl-2-Phenyl-Propylamine*

By RAYMOND P. AHLQUIST, BERTHA HOBENSACK,† and R. A. WOODBURY‡

**The pharmacological action of N-methyl-N-cinnamyl-2-phenyl-propylamine (Cinnamyl Vonedrine—Merrell) has been investigated. It produces peripheral vasodilation and increases the cardiac output in the intact dog when injected intravenously. It also produces vasodilation in the vascular beds supplied by the femoral, carotid, renal and uterine arteries when injected intra-arterially. Cinnamyl Vonedrine increases the coronary flow and stimulates the myocardium of the perfused rabbit heart. Its action upon the intestine and uterus is more variable. Both structures may be stimulated or inhibited depending upon the dose administered or the concentration applied. The isolated rabbit uterus is stimulated with low concentrations and inhibited by high concentrations while the isolated rat uterus is usually inhibited by any effective concentration.**

N-METHYL-2-PHENYL-PROPYLAMINE (Vonedrine—Merrell) is a sympathomimetic pressor amine (1). The addition of a cinnamyl group, to form N-methyl-N-cinnamyl-2-phenyl-propylamine (hereinafter called Cinnamyl Vonedrine), produces a depressor agent. The structural formulas are given below.




Vonedrine Cinnamyl Vonedrine

This paper reports the results of a pharmacological study of the cardiovascular and smooth muscle effects of Cinnamyl Vonedrine in experimental animals. The amine was used in the form of its water-soluble hydrochloride salt and its effects were compared to those of ephedrine, epinephrine, and the parent substance Vonedrine.

## METHODS

**Cardiovascular.**—The studies on the intact cardiovascular system were done on dogs anesthetized with morphine sulfate, 10 mg. per Kg., subcutaneously, followed by pentobarbital sodium, 10–20 mg. per Kg., intravenously. Arterial pressure was recorded by either an ordinary mercury manometer or a Hamilton optical manometer (2) from the carotid or femoral artery. In some of the dogs the aortic pressure pulse was recorded optically on a high-speed camera by means of a metal sound inserted through the carotid artery into the arch of the aorta. Stroke volume and minute output of the heart were calculated from these pressure pulses by the method of Hamilton and Remington (3).

Blood flow in various vascular beds of most of the animals was measured directly by means of a Shipley optical rotameter (4) interposed in the appropriate artery. These dogs were heparinized, 10 mg. per Kg., every two hours. The vascular beds studied were those supplied by the femoral, carotid, uterine, and renal arteries.

In order to determine the peripheral vascular effects of Cinnamyl Vonedrine, the vasomotor resistance ( $VR$) was calculated by the formula (5),

$$VR = \frac{P - 20}{F}$$

in which $P$ is the arterial pressure in mm. Hg, and $F$ the volume arterial inflow in cc. per minute. The total vasomotor resistance ($R_t$) was calculated by the same formula with $F$ as the cardiac output in cc. per second per square meter of body surface. $P - 20$ was used since the $P/F$ ratio changes abruptly at a pressure of about 20 mm. Hg. A decrease in $VR$ indicates vasodilation and an increase indicates vasoconstriction.

Atropine sulfate, 0.5 mg. per Kg., was used as an anticholinergic agent in some of the animals and Dibenamine HCl[1] (6), 20 mg. per Kg., was used as a sympatholytic agent.

The isolated heart of the rabbit or cat was perfused by the method of Langendorff. The myocardial contractions were recorded with an optical lever and the coronary inflow measured with the optical rotameter. The drugs were injected into the coronary inflow.

**Smooth Muscle.**—The activity of the isolated intestine and uterus was recorded by the method of Magnus.[2] Rabbit ileum and uterus, guinea-pig ileum, and rat uterus were used. The rats and guinea pigs from which the uterine strips were obtained were

* Received April 19, 1949, from the Department of Pharmacology, University of Georgia School of Medicine, Augusta, Ga.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.
This study was supported by a grant from The William S. Merrell Co., Cincinnati, Ohio.
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[1] The Dibenamine was kindly supplied by Smith, Kline and French Laboratories, Philadelphia, Pa.
[2] The saline solutions used had the following composition. For gut and uterus: NaCl 0.9%, KCl 0.042%, $CaCl_2$ 0.024%, $MgCl_2$ 0.01%, $NaHCO_3$ 0.03%, and Dextrose 0.1%. For perfused heart: NaCl 0.9%, KCl 0.042%, $CaCl_2$ 0.024%, $NaHCO_3$ 0.015%, and Dextrose 0.1%.

old breeding stock in various stages of estrus. Most rabbits were pretreated for five to seven days with stilbestrol, 0.003 mg. per Kg. Pitressin was used as a spasmogenic agent on the rabbit uterine strips and histamine on the guinea-pig intestinal strips.

The activity of the intact intestine and uterus (pregnant and nonpregnant) was recorded in anesthetized dogs by means of a water-filled balloon and optical manometer system. The balloons were inserted into the lumen of the ileum or uterus through stab wounds after exposure through a midline abdominal incision.

## RESULTS

**Cardiovascular.**—The effects of Cinnamyl Vonedrine on the intact cardiovascular system are illustrated in Fig. 1. With an intravenous dose of 5 mg. per Kg. the arterial pressure fell. Associated with the depressor effect was an increase in heart rate, stroke volume, and cardiac output. The pulse pressure was usually increased during the depressor phase. The fall in arterial pressure was due primarily to peripheral vasodilation as shown by the marked decrease in $VR$ and $R_t$. Following the depressor phase there was usually a slight pressor phase together with a slight decrease in heart rate.

The minimal intravenous dose that would produce a significant depressor effect was found to be about 0.1 mg. per Kg. Cinnamyl Vonedrine was rapidly absorbed from the intestinal tract. A dose of 25 mg. per Kg., introduced into the ileum through a catheter, produced an increase in heart rate and peripheral blood flow together with a small fall in arterial pressure, occurring within fifty seconds and persisting for two or more hours.

Intra-arterial injections of Cinnamyl Vonedrine produced vasodilation in all of the vascular beds studied. Table I illustrates the type of results obtained.

Atropine had no significant effect on any of the cardiovascular effects of Cinnamyl Vonedrine. Dibenamine HCl, administered intravenously ninety minutes before, increased and prolonged the depressor and vasodilator effects of Cinnamyl Vonedrine.

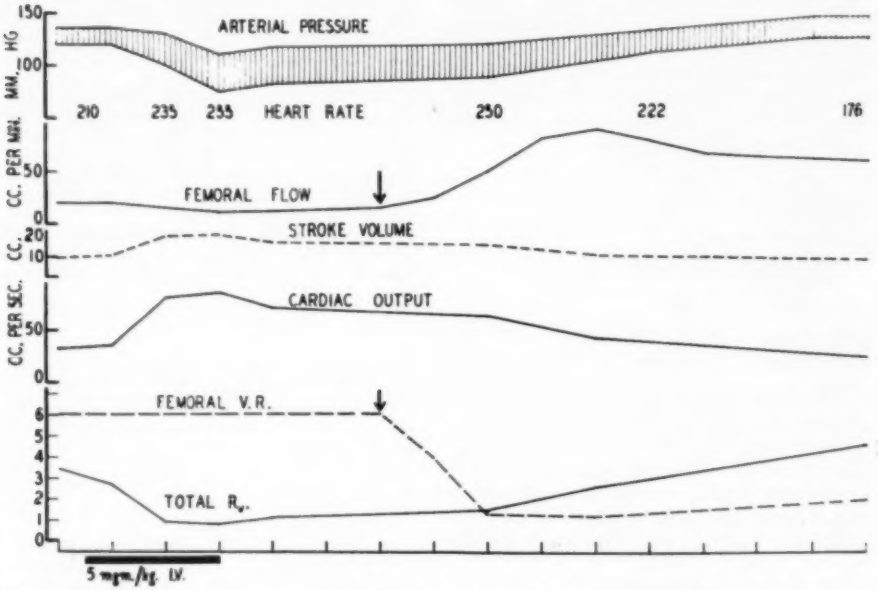


Fig. 1.—The hemodynamic responses of an anesthetized, atropinized dog to an intravenous dose of Cinnamyl Vonedrine.

Records from above downward: systolic, diastolic, and pulse pressures; heart rate in beats per min.: femoral artery volume flow; stroke volume in cc. per square meter body surface; cardiac output in cc. per sec. per square meter; femoral vasomotor resistance; total vasomotor resistance; time marks at ten-second intervals.

The Cinnamyl Vonedrine was injected into a femoral vein during the indicated twenty-five seconds. The arrows indicate the point at which the drug had passed through the flowmeter and into the leg to bring about its typical peripheral vascular effect.

The isolated myocardium was stimulated by doses of Cinnamyl Vonedrine from 0.01 to 1 mg. The stimulation was not as striking as that produced by epinephrine and was manifested mainly as an increase in the amplitude of contraction. Doses greater than 1 mg. produced myocardial inhibition. The coronary inflow was increased by any dose tested.

**Isolated Ileum and Uterus.**—The most important results are given in Table II. It will be seen that with but one exception Cinnamyl Vonedrine produced inhibition of the tissues tested. In a concentration of about 0.001 *M* it had a marked inhibitory and antispasmodic effect as illustrated in Fig. 2. The rabbit uterus was usually stimulated by a concentration of 0.0001 *M*. Vonedrine produced only stimulation with any effective concentration on all of the structures tested.

**Intact Ileum and Uterus.**—The effects of intravenous or oral doses of Cinnamyl Vonedrine on these structures were quite insignificant. A 5 mg. per Kg. intravenous dose produced inhibition of the ileum lasting about one minute while a 25 mg. per Kg. dose given into the small intestine had no measurable effect. The amplitude of normal uterine contractions was diminished very slightly by intravenous doses up to 10 mg. per Kg. The responses of the ileum and uterus to acetylcholine, histamine, and posterior pituitary extracts were apparently not modified by Cinnamyl Vonedrine in the intact anesthetized dog.

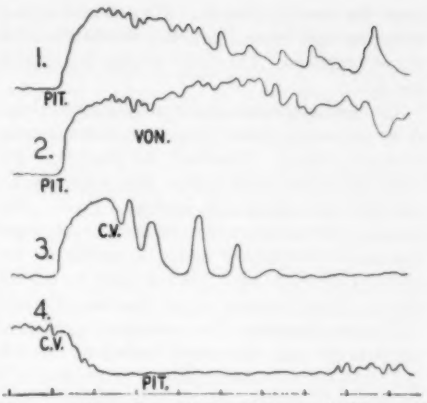


Fig. 2.—The effect of Vonedrine and Cinnamyl Vonedrine on the isolated rabbit uterus.

(*1*) Application of 1 unit Pitressin. (*2*) Pitressin 1 unit followed by Vonedrine 0.001 *M* (final bath concentration). (*3*) Pitressin 1 unit followed by Cinnamyl Vonedrine 0.001 *M*. (*4*) Cinnamyl Vonedrine 0.001 *M* followed by Pitressin 1 unit. Time marks at thirty-second intervals.

## DISCUSSION

Two theories as to the mechanism of action of Cinnamyl Vonedrine can be advanced. It may act either as a sympathomimetic depressor and inhibitory agent or it may have a nonspecific inhibitory effect such as that of papaverine.

It is known that certain modifications in the chemical structure of the sympathomimetic amines yield compounds which are primarily depressor and inhibitory. Some of these are certain N-alkyl homologs of epinephrine or Sympatol (7–9), ethyl nor-epinephrine (10), methoxy phenyl-*n*-propylamines (11), *p*-*n*-alkyloxyphenylethylamines (12), and cinnamyl ephedrine (13). As pointed out in a recent paper from this laboratory (14) these depressor amines should have the following general pharmacological properties. They should produce vasodilation and usually

TABLE I.—THE PERIPHERAL VASCULAR ACTION OF INTRA-ARTERIAL INJECTIONS OF CINNAMYL VONEDRINE

| Dog | Vascular Bed | Dose, Mg./Kg. | Arterial Pressure, C[a] | Arterial Pressure, X[b] | Vasomotor Resistance, C[a] | Vasomotor Resistance, X[b] |
|---|---|---|---|---|---|---|
| 1 | Femoral | 0.01 | 133/95 | 133/95 | 4.9 | 3.2 |
| | | 0.1 | 130/95 | 125/90 | 4.6 | 1.7 |
| 2 | Femoral | 0.01 | 150/110 | 150/110 | 1.3 | 1.1 |
| | | 0.1 | 150/110 | 140/100 | 2.1 | 1.0 |
| 3 | Femoral | 0.5 | 150/110 | 150/100 | 21.0 | 2.3 |
| 4 | Uterine | 0.5 | 105/60 | 100/55 | 3.7 | 2.1 |
| 5 | Renal | 1.0 | 110/75 | 100/70 | 2.1 | 1.6 |
| 6 | Carotid | 1.0 | 135/110 | 125/90 | 2.3 | 2.1 |

[a] Control value.
[b] At maximum increase in blood flow following injection.

TABLE II.—COMPARATIVE EFFECTS OF CINNAMYL VONEDRINE, VONEDRINE, EPHEDRINE, AND EPINEPHRINE ON ISOLATED SMOOTH MUSCLE[a]

| Muscle | Cinnamyl Vonedrine | Vonedrine | Ephedrine | Epinephrine |
|---|---|---|---|---|
| Rabbit Ileum (Inhibition of normal activity) | 0.00001 *M*[b] | Stim. | 50–200 | 0.001–0.0001 |
| Guinea-pig Ileum (Inhibition of histamine-induced spasm) | 0.00001 *M* | Stim. | 5–10 | 0.01–0.001 |
| Rabbit Uterus (Stimulation) | 0.0001 *M* | 1 | 0.1–1 | 0.001–0.0001 |
| Rabbit Uterus (Inhibition of Pitressin spasm) | 0.001 *M* | Stim. | 5–10[c] | Stim. |
| Rat Uterus (Inhibition of normal activity) | 0.0005 *M* | Stim. | 0.1 | 0.001–0.0001 |

[a] All of these comparisons were made on a molar basis. Cinnamyl Vonedrine has been arbitrarily given the value of one. The whole numbers indicate "times less active" and the decimals indicate how much less is necessary to produce a response approximately equal to that of Cinnamyl Vonedrine.
[b] The approximate minimal final bath concentration necessary to produce the indicated effect.
[c] Obtained on only a few of the uterine strips tested. Ephedrine usually produced only stimulation.

lower the arterial pressure. They should inhibit the uterus and bronchi and they should *stimulate* the myocardium. They may or may not inhibit the gut.

The cardiovascular effects of Cinnamyl Vonedrine apparently classify it as a sympathomimetic depressor amine. However, its effects on the ileum and uterus tend to show that it can also be classified as a nonspecific inhibitory agent. For example, if Cinnamyl Vonedrine were a sympathomimetic inhibitory amine it might be expected to have its most marked effect on the rat uterus. This, however, is not the case. Certain differences between the inhibitory action of epinephrine and Cinnamyl Vonedrine on the isolated ileum were also apparent. The response to Cinnamyl Vonedrine was usually delayed (up to two minutes) following the application of submaximal concentrations, whereas the response to epinephrine was always more or less instantaneous. It was very difficult to wash out the inhibitory effect of Cinnamyl Vonedrine.

While it is probable that Cinnamyl Vonedrine has both of the postulated mechanisms of action, further study will be necessary to obtain a final answer. The therapeutic potentialities of Cinnamyl Vonedrine are now under investigation.

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# Some Toxicological Properties of Surface-Active Agents*

By S. S. HOPPER, H. R. HULPIEU, and VERSA V. COLE†

**The toxicological properties of several surface-active agents have been investigated. It has been demonstrated that acute toxicity by vein and by mouth are unrelated. Neither are acute and subacute oral toxicity related quantitatively. Blood pressure effects have not been found to be important factors in intravenous toxicity.**

DURING recent years, there have been a large number of studies done on the toxicity of various surface-active agents. The literature reviewed below pertains chiefly to the agents listed in this study. Smyth, Seaton, and Fischer (1) have described the acute and chronic toxicities by mouth and toxicities by skin absorption of the "tergitol" penetrants. M. and P. Garcia de Jalon (2) have shown that Igepon T may be given in intravenous daily doses of 10 milligrams per rabbit, and 5 milligrams per guinea pig without any change in weight or manifestation of toxic symptoms. Benaglia, Robinson, Utley, and Cleverdon (3) gave Aerosol O.T., 0.87 Gm. per Kg. per day to rats in food. The rats survived a six month experimental period with only occasional diarrhea. Vivino and Koppanyi(4) gave 3 successive generations of rats one tenth per cent Emulsept as drinking water and found no ill effect. Smyth, Carpenter, and Shaffer (5, 6) reported on the toxicity of polyethylene glycols of various molecular weights. They used male albino rats and gave injections by the intraperitoneal route. Polyethylene glycol "400" gave an $LD_{50}$ of 32.8 and Carbowax "1000," 15.57 Gm. per Kg.

* Received October 15, 1948, from the departments of Public Health and Pharmacology, Indiana University School of Medicine, Indianapolis.

† The authors wish to thank the following companies for supplying materials: American Cyanamide and Chemical Corporation (Aerosol O.S. and Aerosol O.T.); Atlas Powder Company (Tween 20 and Tween 80); Carbide and Carbon Chemicals, Corporation (Amine 220, Carbowax "1000" distearate, Carbowax "1000" monostearate, Polyethylene glycol "400" distearate, and Polyethylene glycol "400" monostearate); Emulsol Corporation (Emcol 888 and Emulsept); General Dyestuff Corporation (Igepal CA and Igepon T); Monsanto Chemical Company (Areskap 100, Aresket 300, Aresklene 400, Santomerse 3 and Santomerse D); Nopco Chemical Company (Monosulf, Nopalcol 4-O, Nopalcol 6-O, Nopalcol 6-L, Nopco 2272-R, and Nopcogen 14-L); Onyx Oil and Chemical Company (CDEA Br, and Tetrosan); L. Sonneborn Sons, Incorporated (Penequik and Sulfatex); Victor Chemical Works (Victawet 41 #E-4170), and Winthrop Chemical Company, Incorporated (Roccal).

Fitzhugh and Nelson (7) reported a two-year chronic toxicity experiment on six surface-active agents. They found that the toxic effects of the surface-active agents were produced by irritation of the gastrointestinal tract. Freeman, Burrill, Li, and Ivy (8) found that large doses of a mixture of alkyl aryl sodium sulfonates produced irritation of the gastrointestinal tract in dogs. The experimental results reported here also showed a relationship between irritation and toxicity by mouth.

In the course of studies on the influence of various surface-active agents on drug action, their toxicological properties were investigated. Acute toxicity both by vein and by mouth were determined, as well as subacute toxicity by mouth and corneal irritation. Since a fairly large number of agents were investigated it was thought that these data would be of value to those interested in surface-active agents.

## EXPERIMENTAL

The commercial name and chemical description of all agents used is given in Table I. All results given are for the active material present. A few of the agents were furnished in solution and corrections for concentration were made. In all toxicity experiments, albino mice of the Harlan strain were used, ranging in weight from 14 to 23 Gm. All doses were given so that 0.5 cc. of solution was given for each 20 Gm. of mouse. The mice were divided into groups of 10 each, matched for sex, and varying doses of each compound given with an increasing increment between doses of 20 per cent or less. The $LD_{50}$ was then calculated from the death rate at the dosages given. The lowest death rate used in any calculation was 3 out of 10, and the highest 7 out of 10. A straight line relationship between dosage and death rate was assumed at these levels. Intravenous toxicity was determined on all compounds studied. The mice were kept for 24 hours after the intravenous injection and all deaths within 24 hours were considered to be caused by the drug. For acute toxicity *per os*, it was impossible, in some instances, to obtain a sufficiently concentrated solution for testing. The mice were kept for 72 hours after a dose of the agent by stomach tube, and all deaths within 72 hours were considered to be due to the surface-active agent. The results on these experiments can be seen in Table II. It can be noted from these results that there appears to be no definite relationship between toxicity by vein and toxicity *per os*.

TABLE I.—COPYRIGHT AND CHEMICAL NAMES OF AGENTS STUDIED

| Agents Studied | Chemical Names |
|---|---|
| **Nonionic** | |
| Carbowax "1000" monostearate | Polyethylene glycol monostearate, high molecular weight |
| Carbowax "1000" distearate | Polyethylene glycol distearate, high molecular weight |
| Igepal CA | Polymerized ethylene oxide condensate |
| Monosulf | Sulfonated castor oil |
| Nopalcol 4-O | Polyglycol oleate |
| Nopalcol 6-O | Polyethylene glycol oleate |
| Nopalcol 6-L | Polyglycol laurate |
| Nopco 2272-R | Sulfated butyl oleate |
| Nopcogen 14-L | Diethyl amido laurate |
| Penequik | Sulfonated ester |
| Polyethylene glycol "400" monostearate | Polyethylene glycol monostearate, low molecular weight |
| Polyethylene glycol "400" distearate | Polyethylene glycol distearate, low molecular weight |
| Tween 20 | Polyoxyethylene ester of sorbitan monolaurate |
| Tween 80 | Polyoxyethylene ester of sorbitan monooleate |
| **Anionic** | |
| Aerosol O.S | Isopropylnaphthalene sodium sulfonate |
| Aerosol O.T | Dioctyl ester of sodium sulfosuccinic acid |
| Areskap 100 | Monobutylphenyl-phenol sodium monosulfonate |
| Aresket 300 | Monobutyl diphenyl sodium monosulfonate |
| Aresklene 400 | Dibutylphenyl-phenol sodium disulfonate |
| Igepon T | $C_{17}H_{33}CON\text{-}CH_3C_2H_4SO_3Na$ |
| Santomerse 3 | Dodecyl benzene sodium sulfonate |
| Santomerse D | Decyl benzene sodium sulfonate |
| Sulfatex | Sodium and potassium salts of a sulfated fatty alcohol amide |
| Victawet 41#E-4170 | Phosphorus containing compound |
| **Cationic** | |
| Amine 220 | 80% 1-hydroxyethyl-2-heptadecenyl glyoxalidine, 20% monooleyl amide of monohydroxyethyl ethylene diamine |
| CDEA Br | Cetyl dimethyl ethyl ammonium bromide |
| Emcol 888 | Polyalkyl naphthalene pyridinium chloride, average molecular weight, 382 |
| Emulsept | Lauric and myristic acid esters of colaminoformylmethyl-pyridinium chloride |
| Roccal | A mixture of high molecular alkyl dimethyl benzyl ammonium chlorides |
| Tetrosan | A mixture of alkyl dimethyl 3,4-dichlorobenzyl ammonium chlorides |

TABLE II.—ACUTE TOXICITY OF SURFACE-ACTIVE AGENTS

| Nonionic Agents | $LD_{50}$ in Mice 24 hr., I.V., Mg./Kg. | $LD_{50}$ in Mice 72 hr., *per os*, Gm./Kg. | Anionic Agents | $LD_{50}$ in Mice 24 hr., I.V., Mg./Kg. | $LD_{50}$ in Mice 72 hr., *per os*, Gm./Kg. | Cationic Agents | $LD_{50}$ in Mice 24 hr., I.V., Mg./Kg. | $LD_{50}$ in Mice 72 hr., *per os*, Gm./Kg. |
|---|---|---|---|---|---|---|---|---|
| Carbowax "1000" monostearate | 870 | [a] | Aerosol O.S. | 120 | 1.90 | Amine 220 | 88 | [a] |
| Carbowax "1000" distearate | 220 | [a] | Aerosol O.T. | 60 | 4.80 | CDEA Br[b] | 50 | 0.60 |
| Igepal CA | 70 | 3.50 | Areskap 100 | 180 | 3.80 | Emcol 888 | 8 | 0.47 |
| Monosulf | 88 | >25.00 | Aresket 300 | 250 | 3.50 | Emulsept | 20 | >2.50 |
| Nopalcol 4-O | 1080 | >25.00 | Aresklene 400 | 200 | 2.20 | Roccal | 16 | 0.34 |
| Nopalcol 6-O | 500 | >25.00 | Igepon T | 350 | 6.63 | Tetrosan | 50 | 2.00 |
| Nopalcol 6-L | 500 | >25.00 | Santomerse 3 | 105 | 2.00 | | | |
| Nopco 2272-R | 63 | 8.75 | Santomerse D | 115 | 2.10 | | | |
| Nopcogen 14-L | 287 | 9.65 | Sulfatex | 587 | >25.00 | | | |
| Penequik | 175 | 5.80 | Victawet 41 ƒE-4170 | 250 | 2.56 | | | |
| Polyethylene glycol "400" monostearate | 250 | [a] | | | | | | |
| Polyethylene glycol "400" distearate | 365 | [a] | | | | | | |
| Tween 20 | 3750 | >25.00 | | | | | | |
| Tween 80 | 5800 | >25.00 | | | | | | |

[a] Unable to obtain a solution concentrated enough for testing.
[b] Cetyl dimethyl ethyl ammonium bromide.

TABLE III.—SUBACUTE TOXICITY OF SURFACE-ACTIVE AGENTS

Number of Mice Dead After Receiving 0.1 $LD_{50}$ *per os* Daily, 6 Days per Week. Ten Mice in Each Test

| | Number of Doses | 5 | 10 | 15 | 20 | 25 |
|---|---|---|---|---|---|---|
| Nonionic | Igepal CA | 0 | 0 | 2 | 3 | 3 |
| | Nopco 2272-R | 1 | 4 | 4 | 6 | 7 |
| | Nopcogen 14-L | 0 | 1 | 1 | 1 | 1 |
| | Penequik | 1 | 3 | 3 | 3 | 4 |
| Anionic | Aerosol O.S. | 2 | 4 | 6 | 7 | 7 |
| | Aerosol O.T. | 5 | 7 | 8 | 8 | .. |
| | Areskap 100 | 3 | 4 | 5 | 5 | 5 |
| | Aresket 300 | 1 | 2 | 3 | 4 | 4 |
| | Aresklene 400 | 1 | 1 | 1 | 1 | 1 |
| | Igepon T | 1 | 5 | 5 | 5 | 5 |
| | Santomerse 3 | 0 | 1 | 1 | 1 | 1 |
| | Santomerse D | 0 | 0 | 1 | 1 | 1 |
| | Victawet 41 ƒE-4170 | 1 | 1 | 2 | 2 | 2 |
| Cationic | CDEA Br | 5 | 8 | 8 | 8 | .. |
| | Emcol 888 | 1 | 3 | 6 | 7 | .. |
| | Roccal | 0 | 1 | 2 | 2 | 2 |
| | Tetrosan | 4 | 9 | 9 | .. | .. |

TABLE IV.—THE EFFECT OF SINGLE DOSES[a] OF SURFACE-ACTIVE AGENTS ON THE BLOOD PRESSURE

| Agents | No. of Dogs | No. of B. P. Observations | Range in Effect on B. P., Mm. of Hg | Average Effect on B. P., Mm. of Hg By Dogs | Average Effect on B. P., Mm. of Hg By Observations |
|---|---|---|---|---|---|
| **Nonionic** | | | | | |
| Igepal CA | 4 | 10 | −32 to −82 | −56 | −50 |
| **Anionic** | | | | | |
| Aerosol O.S. | 3 | 3 | +12 to −28 | −12 | −12 |
| Aerosol O.T. | 3 | 4 | −16 to −76 | −39 | −44 |
| Aerskap 100 | 4 | 8 | −8 to −32 | −24 | −24 |
| Aresket 300 | 4 | 6 | −18 to −36 | −29 | −30 |
| Aresklene 400 | 7 | 17 | −16 to −64 | −36 | −33 |
| Santomerse 3 | 4 | 5 | 0 to −30 | −9 | −10 |
| Santomerse D | 3 | 5 | +20 to −12 | +5 | −1 |
| **Cationic** | | | | | |
| CDEA Br | 3 | 4 | −40 to −74 | −52 | −54 |
| Emcol 888 | 3 | 4 | 0 to −16 | −5 | −4 |
| Emulsept | 3 | 3 | +10 to −12 | +3 | +3 |
| Roccal | 3 | 4 | 0 to −20 | −10 | −10 |

[a] One-tenth of $LD_{50}$ for mice by vein.

TABLE V.—THE EFFECT OF SURFACE-ACTIVE AGENTS ON BLOOD PRESSURE AND SURVIVAL IN THE DOG[a]

| Dog No. | Total Dose, Cc./Kg. | Change in B. P., Mm. of Hg | Final Outcome |
|---|---|---|---|
| | | Igepal CA | |
| 1 | 6.5 | −100 | Death |
| 2 | 3.2 | −94 | Death |
| 3 | 6.1 | −122 | Death |
| 4 | 3.6 | −130 | Death |
| 5 | 9.0 | −82 | Return to normal |
| 6 | 5.3 | −120 | Death |
| | | Santomerse D | |
| 1 | 10.0 | +30 | Return to normal |
| 2 | 10.0 | +24 | Unchanged |
| 3 | 10.0 | −40 | Unchanged |
| 4 | 10.0 | +36 | Return to normal |
| | | Cetyl Dimethyl Ethyl Ammonium Bromide | |
| 1 | 7.4 | +10 | Returnto normal |
| 2 | 9.0 | +6 | Return to normal |
| 3 | 9.0 | −28 | Return to normal |
| 4 | 10.0 | −28 | Sacrificed |

[a] Dose 0.1 cc./Kg. per minute of a 1% solution.

some agents appear to show a higher toxicity in divided doses than in a single dose, in particular, Aerosol O.T., cetyl dimethyl ethyl ammonium bromide, and Tetrosan.

The effects of some of these agents on blood pressure were studied on dogs by the single dose method using one-tenth of the $LD_{50}$ for mice by vein. Those compounds showing a consistent fall in blood pressure are Igepal, Aresket 300, Aresklene 400, and cetyl dimethyl ethyl ammonium bromide. The effect of these compounds on blood pressure was not altered by atropine. The other agents had practically no effect on blood pressure. The results are shown in Table IV. The effect of continuous injection on blood pressure was studied on 3 agents, one each of the nonionic, cationic, and anionic types (Table V). Igepal showed a continuous fall resulting in death in 5 of 6 animals. The others showed irregular effects with return to normal after stopping the administration of the surface-active agent. There is no relationship, apparently, between the effect of a single dose and continuous administration of a smaller dose. Blood pressure does not seem to be an important factor in toxicity.

TABLE VI.—IRRITATION PRODUCED BY SURFACE-ACTIVE AGENTS ON THE RABBIT'S CORNEA

| Agent | pH of Solution | Photophobia | Discharge | Inflammation | Edema | Cloudy Cornea | Total |
|---|---|---|---|---|---|---|---|
| **Nonionic** | | | | | | | |
| Igepal CA | 6.5 | 0 | 2 | 4 | 0 | 0 | 6 |
| Monosulf | 6.8 | 0 | 0 | 1 | 0 | 0 | 1 |
| Nopalcol 4-O | 6.9 | 0 | 0 | 1 | 0 | 0 | 1 |
| Nopalcol 6-O | 6.9 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nopalcol 6-L | 7.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| Nopco 2272-R | 6.5 | 0 | 0 | 2 | 0 | 0 | 2 |
| Nopcogen 14-L | 9.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tween 20 | 6.4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Tween 80 | 5.6 | 0 | 0 | 0 | 0 | 0 | 0 |
| **Anionic** | | | | | | | |
| Aerosol O.T. | 6.0 | 0 | 0 | 6 | 0 | 0 | 6 |
| Areskap 100 | 8.3 | 0 | 0 | 2 | 0 | 0 | 2 |
| Aresket 300 | 6.5 | 0 | 1 | 6 | 0 | 0 | 7 |
| Aresklene 400 | 8.8 | 0 | 2 | 4 | 0 | 0 | 6 |
| Igepon T | 7.2 | 0 | 0 | 2 | 0 | 0 | 2 |
| Santomerse 3 | 6.1 | 2 | 0 | 8 | 0 | 0 | 10 |
| Santomerse D | 7.2 | 3 | 4 | 8 | 0 | 0 | 15 |
| Sulfatex | 6.4 | 0 | 0 | 0 | 0 | 0 | 0 |
| Victawet 41 #E-4170 | 6.7 | 0 | 0 | 6 | 3 | 0 | 9 |
| **Cationic** | | | | | | | |
| CDEA Br | 6.1 | 0 | 2 | 8 | 9 | 12 | 31 |
| Emcol 888 | 4.0 | 1 | 4 | 8 | 6 | 16 | 35 |
| Emulsept | 4.8 | 0 | 0 | 0 | 0 | 0 | 0 |
| Roccal | 7.4 | 2 | 4 | 8 | 6 | 16 | 36 |
| Tetrosan | 5.2 | 2 | 4 | 8 | 0 | 0 | 14 |

Concentrations used in all instances was 1%.

Scale for evaluating the irritation:

| | |
|---|---|
| Photophobia | 1 |
| Discharge | 1 |
| Mild inflammation | 1 |
| Severe inflammation | 2 |
| Edema | 3 |
| Cloudy cornea | 4 |

Observations at:
5 minutes
10 minutes
1 hour
24 hours

Also, the toxicity of two compounds by the intraperitoneal route (5, 6) was in reverse order to our findings by vein.

For our studies on subacute toxicity, mice were given one-tenth the $LD_{50}$ *per os* daily six days per week. Experiments were terminated after 25 doses. From these results, shown in Table III,

The irritation of the various surface-active agents on the rabbit's cornea was rated according to the method of Whitehill (9). Data are presented in Table VI. Observations were made at five minutes, ten minutes, one hour, and twenty-four hours. The number of positive observations were multiplied by the rating in the scale for evaluating irritation

TABLE VII.—RELATIONSHIP BETWEEN ACUTE TOXICITY AND IRRITATION

| Agents in Order of Toxicity | $LD_{50}$ in Mice Gm./Kg. by Mouth | Irritation in Eye | No. of Agents | Range Irritation | Average Irritation |
|---|---|---|---|---|---|
| Roccal | 0.34 | 36 | | | |
| Emcol 888 | 0.47 | 35 | 3 | 31–36 | 34 |
| CDEA Br | 0.60 | 31 | | | |
| Santomerse 3 | 2.00 | 10 | | | |
| Tetrosan | 2.00 | 14 | | | |
| Santomerse D | 2.10 | 15 | 5 | 6–15 | 11 |
| Aresklene 400 | 2.20 | 6 | | | |
| Victawet 41 | 2.56 | 9 | | | |
| Igepal CA | 3.50 | 6 | | | |
| Aresket 300 | 3.50 | 7 | | | |
| | | | | 2–7 | 5 |
| Areskap 100 | 3.80 | 2 | | | |
| Aerosol O.T. | 4.80 | 6 | | | |
| Igepon T | 6.63 | 2 | | | |
| | | | 2 | 2 | 2 |
| Nopco 2272-R | 8.75 | 2 | | | |
| Nopcogen 14-L | 9.65 | 0 | | | |
| Monosulf | >25.00 | 1 | | | |
| Nopalcol 4-O | >25.00 | 1 | | | |
| Nopalcol 6-O | >25.00 | 0 | | | |
| | | | 8 | 0–1 | <1 |
| Nopalcol 6-L | >25.00 | 0 | | | |
| Tween 20 | >25.00 | 0 | | | |
| Tween 80 | >25.00 | 0 | | | |
| Sulfatex | >25.00 | 0 | | | |

to give the number appearing in the chart. The most irritating agents were found to be in the cationic group. In general, the least irritating were found in the nonionic group. However, the necessity for testing each individual compound is illustrated by the variations within each group. There is no relationship between acute toxicity by vein and irritation; however, there is some relationship between acute toxicity by mouth and irritation. This is shown by the analysis of the data obtained on twenty-two agents (Table VII). With these agents, it was possible to determine a definite toxicity or it was possible to give 25 Gm. per Kg. without obtaining an $LD_{50}$. It can be seen that those compounds having an irritation rating of 1 or less have a low toxicity by mouth and those having a rating of over 30 have a high toxicity.

## CONCLUSIONS

(1) With the surface-active agents studied, acute toxicity by vein and by mouth are not related.

(2) Acute oral toxicity and subacute oral toxicity are not related quantitatively.

(3) Blood pressure effects are not an important factor in intravenous toxicity.

(4) There seem to be a relationship between acute oral toxicity in mice and irritation produced in the rabbit's eye.

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# A Summary of the Pharmacological and Toxicological Properties of Anthallan (3-Di(n-butyl)-aminomethyl-4,5,6-trihydroxy-phenylphthalide)*

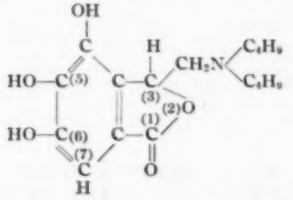
EDITED by LLOYD W. HAZLETON

**The chemistry of Anthallan is reviewed and a series of pharmacological and toxicological experiments are described and the results reported. Pharmacologically, Anthallan is relatively inert and its toxicity is of a low order. The results of the investigations reported indicate that prolonged large doses may be employed without toxic effect.**

ANTHALLAN[1] has achieved considerable clinical usage and several clinical publications on it are available (1–4), but the literature is almost devoid of information concerning its pharmacological and toxicological properties. The present report is designed to combine and coordinate a series of experimental studies which have been conducted on Anthallan alone and in combination with aminophylline[1] (5) but have not previously been published. Authors contributing to this study are Frederic Damrau, New York City; Lloyd W. Hazleton, Hazleton Laboratories; Gerhard Katz, Lynchburg, Va.; Theodore Koppanyi, Georgetown University; S. Loewe, University of Utah, and Nicholas M. Molnar, Molnar Laboratories, each of whom has reviewed this presentation of his contribution.

## CHEMISTRY

Anthallan is a new synthetic organic substance represented by the formula 3′-di(*n*-butyl)aminomethyl - 4,5,6 - trihydroxy - benzo - (1,2 - c)furan-1′(3′)-one or 4,5,6-trihydroxyphthalidyl-di(*n*-butyl)-amino-methane or 3-di(*n*-butyl)aminomethyl-4,5,6-trihydroxy-phenylphthalide.



Anthallan ($C_{17}H_{25}O_6N$) is the lactone of beta-gallic acid-ethanol-alpha-di(*n*-butyl)amine. It is synthesized, according to U. S. Patent No. 2,268,990, by condensing di(*n*-butyl)-aminoacetaldehyde or its acetal with gallic acid under dehydrating conditions. The drug is purified by fractionate precipitation of the base from an acid aqueous solution of the hydrochloric salt with the aid of a moderate surplus of aqueous ammonia in the presence of ammonium acetate buffer. A pure product is obtained at *p*H 6.0.

Anthallan is a grayish to light brownish powder of moderately bitter taste. It is poorly soluble in water and alkali; soluble in alcohol, ether, and various other organic solvents. The molecular weight is 323.2. The melting point is 52.5°.

Numerous salts of the Anthallan base have been prepared by customary methods. Outstanding among them because of adequate water solubility are the hydrochloride and the gluconate.

## PHARMACOLOGY

The following series of pharmacological and toxicological experiments were reported:

LOEWE: For mice, the acute oral $LD_{50}$ of Anthallan base in propylene glycol was 2.70 Gm./Kg., in tragacanth suspension 3.23 Gm./Kg. The acute $LD_{50}$ of an aqueous solution of the hydrochloride in mice was 1.08 Gm./Kg. subcutaneously, 0.30 Gm./-Kg. intraperitoneally, and 0.08 Gm./Kg. intravenously.

KOPPANYI: Propylene glycol solutions of Anthallan injected intravenously or intramuscularly produced a slight decrease in blood pressure, which tended to return to normal if the doses did not exceed 20.0 mg./Kg. In localizing the vasodepressor action the following factors were reported:

(*a*) Vasodepression does not appear to be due to central action because, in two dogs, in which the entire nervous system was destroyed and the blood pressure maintained by continuous infusion of 7.0 gum acacia, Anthallan hydrochloride still induced vasodepression.

(*b*) Vasodepression is apparently not due to action on the cholinergic vasodilator endings for it is not prevented by the administration of suitable doses of atropine sulfate.

(*c*) To determine whether the action might be due to direct effect on the smooth muscle of the blood vessels, six turtle hearts were perfused by the Trendelenburg method. Doses of from 10.0 to 20.0 mg./Kg. of Anthallan hydrochloride perfused through the heart produced no significant change in the rate of flow of the perfusate. In four turtles there was no change, in two there was an increase of from 16 to 18 drops per minute. From this experi-

* Received April 7, 1949, from the Hazleton Laboratories, Falls Church, Va.

[1] Anthallan and its combination with aminophylline, which is also in clinical use under the name of Anthaphylline, are both products of the Medico-Chemical Corporation of America, New York.

ment it was concluded that Anthallan does not possess a nitrite-like action on coronary vessels.

In a series of experiments, dogs and cats were anesthetized with sodium pentobarbital. They were vagotomized and blood pressure was recorded from the carotid artery by means of a mercury manometer. Respiration was recorded from a tracheal canula and Marey tambour. Fixed electrodes were attached to the peripheral end of the divided left vagus.

In six dogs and six cats the administration of Anthallan did not interfere with the cardiac slowing or standstill due to faradic stimulation of the vagus nerve. Neither did Anthallan affect the apneic pause induced by stimulation of the central end of the vagus. There was practically no change in the response to standard doses of acetylcholine, irrespective of the dose of Anthallan, provided the blood-pressure level was not lowered excessively. In these animals Anthallan exerted no significant effect on the pressor response to epinephrine hydrochloride or stimulation of the divided end of the splanchnic nerve.

The effects of Anthallan base and hydrochloride on intact unanesthetized animals are summarized in Table I from a report by Koppanyi. It should be noted that copious defecation follows either intravenous or oral administration in both dogs and cats. Nausea and vomiting were induced only by oral administration of the hydrochloride and are assumed to be due to local irritation. The only other observable action from the doses studied was a "sham rage" reaction following intravenous administration to cats. In the absence of any other evidence of central nervous system stimulation it is possible that this is a reflex action, perhaps vasosensory. All of the animals in Table I were observed for at least one month following the experiments and there was no evidence of toxicity other than transient hemoglobinuria in those receiving propylene glycol as a solvent of Anthallan base.

TABLE I.—EFFECTS OF INTRAVENOUS AND ORAL ADMINISTRATION OF ANTHALLAN HYDROCHLORIDE IN DISTILLED WATER AND THE BASE IN PROPYLENE GLYCOL TO UNANESTHETIZED CATS AND DOGS[a]

| Number | Dose, Mg./Kg. | Preparation | Nausea | Vomiting | Defecation | Other |
|---|---|---|---|---|---|---|
| | | | Dogs—Intravenous | | | |
| 12 | 25 | HCl | 0 | 0 | 3 | .... |
| 12 | 50 | HCl | 0 | 0 | 7 | .... |
| 4 | 25 | Base | 0 | 0 | 4 | .... |
| 5 | 50 | Base | 0 | 0 | 5 | .... |
| | | | Cats—Intravenous | | | |
| 6 | 25 | HCl | 0 | 0 | ... | Sham rage in 9 |
| 6 | 50 | HCl | 0 | 0 | 5 | |
| 6 | 25 | Base | 0 | 0 | ... | Milder excitement, convulsive |
| 3 | 50 | Base | 0 | 0 | 6 | movements in 2 |
| | | | Dogs—Oral | | | |
| 6 | 50 | HCl | 4 | 4 | 6 | .... |
| 1 | 100 | HCl | | | | |
| 5 | 50 | Base | 0 | 0 | 5 | .... |
| | | | Cats—Oral | | | |
| 4 | 50 | HCl | 2 | 2 | 4 | .... |
| 1 | 100 | HCl | | | | |
| 6 | 50 | Base | 0 | 0 | 6 | .... |

[a] Observations other than those tabulated include excitement, convulsions, depression, ataxia, loss of righting reflex, knee jerk, pupil size, and light and corneal reflexes. Reported by Koppanyi.

## ANTIHISTAMINIC PROPERTIES

KATZ: The following series of studies in cats were directed at evaluating qualitatively the antihistaminic properties of Anthallan as measured by the histamine depressor response in blood pressure. Adult cats of either sex under Dial-Urethane anesthesia were used. Both vagi were sectioned, and the mean arterial blood pressure was recorded by means of a mercury manometer. Histamine injections were made through a canula inserted into the external jugular vein. Intravenous infusion of Anthallan was performed with a constant flow system into the right femoral vein, with the right femoral artery ligated. From the results obtained on 35 cats, this author concluded that Anthallan, given as gluconate in aqueous solution by continuous intravenous infusion or as the base in propylene glycol by stomach tube or intravenously, raises the threshold sensitivity of the blood pressure to histamine (0.05 to 0.1 μg. total) and is also capable of depressing the histamine response in the less sensitive multiple threshold range.

When Anthallan gluconate in Tyrode's solution was infused at rates of 0.3 to 1.0 mg./Kg./min. over periods of from ten to thirty minutes, the histamine threshold was raised by 50% to 500%. A distinct histaminolytic effect was observed when Anthallan in propylene glycol was given intramuscularly in doses between 25.0 and 50.0 mg./Kg.

The diminutions of histamine sensitivity after Anthallan were greater than spontaneous fluctuations in histamine sensitivity, and the sensitivity returned to normal, or near normal, after discontinuation of infusion or after the effect of intramuscular or oral administratration had passed its peak.

The action of Anthallan in antagonizing threshold doses of histamine is constant and reproducible, and was observed in one dog, in addition to 24 cats. The Anthallan effect upon the response to higher doses of histamine is less regular.

The only direct effect of Anthallan upon circulation was a moderate vasodepression. That this action of Anthallan is not responsible for the antihistamine activity is established by: (*a*) the depressor effect of Anthallan was not always present during the Anthallan-induced increase in histamine threshold sensitivity; (*b*) in some cases the depressor effects of Anthallan were of short duration and were outlasted by the increase in histamine threshold sensitivity; and (*c*) in some experiments the histamine threshold sensitivity recovered prior to recovery of the blood pressure.

KOPPANYI: The following is a summary of experiments directed at an evaluation of the antihistaminic and antiallergic properties of Anthallan: in 24 dogs and 12 cats the intravenous administration of Anthallan hydrochloride in divided dosage totaling up to 50.0 mg./Kg., or intramuscular administration of Anthallan base in comparable doses, did not appreciably affect the vasodepressor effects of histamine, provided the mean blood-pressure level was not decreased excessively. Koppanyi used doses of 30.0 μg. total, 0.15 cc. of 1:5000 for dogs and 10.0 μg. total, 0.1 cc. of 1:10,000 for cats. These doses approximate the maximum which can be antagonized by antihistaminic drugs (6, 7). Occasionally, particularly in the cats, there was an apparent antihistaminic effect, but this was observed only in cases in which the Anthallan had produced an unusually low mean blood-pressure level. In these animals, after subsequent administration of additional Anthallan at a slower intravenous rate, the blood pressure returned to normal and the antihistaminic effects were no longer evident.

In four dogs the medullary respiratory center was destroyed and continuous artificial respiration was maintained for the duration of the experiment. Intrathoracic pressure was recorded by means of a large needle inserted into the thoracic cavity and connected to a Marey tambour. An increase in the intrathoracic pressure was taken as a manifestation of bronchial dilation, while a decrease in intrathoracic pressure was taken as evidence of bronchial constriction. Under these conditions the injection of Anthallan produced no visible changes in the intrathoracic pressure. Doses of Anthallan up to 50.0 mg./Kg. did not affect the bronchial dilation brought about by epinephrine or the bronchial constriction induced by histamine or acetylcholine.

To study the antiallergic and antihistaminic properties of Anthallan in intact animals, two series of tests were conducted:

(*a*) Thirty-six guinea pigs were sensitized by the subcutaneous administration of 1.0 cc. of horse serum. Eighteen of these animals were given daily doses of 20.0 mg./Kg. of Anthallan administered hypodermically. The remaining 18 received comparable doses of physiological saline solution. The daily injections were conducted for fourteen days, and on the fifteenth day one hour after the daily dose each animal received a challenge dose of 1.0 cc. of horse serum intracardially. This dose killed 15 of 18 Anthallan-treated animals and 13 of 18 control animals.

(*b*) To evaluate the acute action of Anthallan, 18 guinea pigs were injected intravenously with 20.0 mg./Kg. of Anthallan hydrochloride. After ten minutes nine animals received 0.5 and nine animals 1.0 mg./Kg. of histamine phosphate intracardially. The mortality was six of nine receiving the smaller dose, and nine of nine receiving the larger dose. Of 18 control animals comparably treated, the mortality was five of nine for the smaller dose, and nine of nine for the larger dose of histamine.

## CHRONIC TOXICITY

DAMRAU and LOEWE: Chronic feeding experiments were conducted in mice, rats, and guinea pigs by Loewe, and in cats by Damrau. Anthallan base was fed daily at various levels with a suitable number of animals serving as controls. A summary of these experiments is presented in Table II. During the feeding experiments an epidemic of diarrhea occurred in the control and high level rats. Rectal smears were negative for the typhoid group

TABLE II.—SUMMARY OF STUDIES ON ANIMALS AFTER CHRONIC FEEDING WITH ANTHALLAN

| Species | Days | Anthallan, Mg./Kg. Daily | No. of Animals | Average Weight of Animals, Gm. | | Degenerative Toxic Reaction, | Pathological Findings | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Organ Involved | | | | | |
| | | | | Start | Finish | No. of Animals | Liver | Kidney | Spleen | Intestine | Heart | Other Organs |
| Mice | 335 | Control | 6 | 17.2 | 26.4 | 2 | 0 | 2 | 0 | 0 | 0 | 0 |
| | | 100 | 6 | 18.2 | 28.6 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| | | 200 | 8 | 17.8 | 26.3 | 3 | 1 | 1 | 1 | 0 | 0 | 0 |
| Rats | 254 | Control | 8 | 144 | 184 | 3 | 0 | 0 | 1 | 3 | 0 | 0 |
| | | 15 | 2 | 145 | 213 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 50 | 2 | 141 | 214 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| | | 200 | 5 | 142 | 200 | 3 | 0 | 1 | 1 | 1 | 0 | 0 |
| Guinea pigs | 204 | Control | 4 | 292 | 815 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 15 | 4 | 264 | 692 | 1 | 1 | 0 | 0 | 0 | 1 | 0 |
| | | 50 | 4 | 271 | 776 | 1 | 1 | 0 | 0 | 0 | 0 | 0 |
| | | 200 | 4 | 277 | 765 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Cats | 183 | Control | 3 | 1800 | 1820 | No specific pathogenesis | | | | | | |
| | | 60 | 3 | 2450 | 2420[a] | No specific pathogenesis | | | | | | |
| | | 120 | 3 | 2070 | 2280 | No specific pathogenesis | | | | | | |

[a] One pregnant and delivered during experiment.

organisms and the opinion was nonspecific enteritis, epidemic type.

At the conclusion of the feeding period the animals were killed by violence and both gross and microscopic examinations conducted. No specific toxic changes in the organs of the rats, mice, and guinea pigs can be attributed to Anthallan. However, there was evidence of traumatic death, and there were morphologic parenchymatous changes in the cells which are evidence of toxic irritation. This incidence of changes has been tabulated in Table II. In 29.0% of the Anthallan-fed animals, these degenerative changes were found, and 28.0% of the control animals showed the same reactions.

During the course of chronic feeding studies of Anthallan on cats the hemoglobin remained normal, the stools were of normal consistency, and there were no changes in the pilomotor reflexes. On gross and microscopic examination, all changes in the organs were due to trauma or to the usual incidence of pathology in untreated animals. No changes due to Anthallan were noted.

## FATE

LOEWE: The fate of Anthallan in experiments in humans and animals was investigated. The fact that in animals (mice) death from lethal doses follows within a few minutes after the oral administration of Anthallan would indicate that Anthallan is rapidly absorbed from the gastrointestinal tract. This was further demonstrated in human subjects in which traces of Anthallan, as identified by Mitchell's Iron Reagent, were demonstrable in the urine as early as fifteen to twenty-five minutes after oral intake. The chemical test is based on the fact that in aqueous solution at about *p*H 6.2 Anthallan yields a reddish purple color with Mitchell's Reagent.

In nine experiments in four rats, four guinea pigs, and a rabbit, all receiving chronic oral Anthallan treatment, blood was collected from the carotid artery two to five hours after a 200.0- to 500.0-mg./-Kg. oral dose of Anthallan. The blood was deproteinized by trichloracetic acid and the filtrate concentrated to original volume at low pressure. In no case were Mitchell-positive substances detected. In a control experiment 15.0 mg. of Anthallan added to 20.0 cc. of horse serum was recovered with satisfactory accuracy. Tests of aqueous extracts from the stools of animals under similar conditions were negative, whereas added Anthallan could be detected in the stools.

From an analysis of the extraction products appearing in human urine after oral intake, 19% to 45% of the introduced Anthallan could be accounted for in the urine. Most of this 19% to 45% of the Anthallan was chemically changed, with only 5.0% or less being recovered as free basic substance. The greater part, about 70% to 80% of the recoverable substances, could be identified only after hydrolysis and appeared to be conjugates of either Anthallan or its cleavage products. Excretion of Anthallan and its cleavage products was found to reach a maximum in the urine within twelve hours after oral intake and to be virtually completed after thirty-four hours. No indication was obtained of excretion via other routes.

## COMBINATION WITH AMINOPHYLLINE

In clinical practice it is sometimes desirable to initiate Anthallan therapy by supplementing the dosage with approximately equal quantities of aminophylline. To determine whether such a combination would remain a physical mixture or might under physiological conditions form a new chemical entity, the following experiments were conducted.

MOLNAR: A powder mixture containing 0.085 Gm. of Anthallan and 0.100 Gm. of aminophylline U. S. P. was used for the following chemical tests:

1. After standing for twenty-four hours in distilled water, a suspension of this mixture was filtered. The residue gave all reactions for identification and purity for Anthallan, while the evaporated filtrate gave appropriate tests for the presence of aminophylline, indicating that no chemical reaction had taken place.

2. A suspension of the mixture after standing for twenty-four hours in artificial gastric fluid was neutralized with ammonia and filtered. Appropriate tests on the residue and filtrate indicated the presence of Anthallan and aminophylline, respectively, except that there was some evidence that Anthallan undergoes slight decomposition, probably due to the presence of pepsin.

3. The same results were obtained when a suspension of the mixture was allowed to stand for twenty-four hours in artificial intestinal fluid, except that there was some evidence that Anthallan undergoes slight decomposition, probably due to the presence of pancreatin.

It is therefore apparent that under the above conditions a mixture of Anthallan and aminophylline remains a physical mixture with no chemical interaction. Slightly reduced recovery of Anthallan after standing in artificial gastric or intestinal fluid indicates that there is some decomposition of the Anthallan.

HAZLETON: The following biological tests were designed to determine the effect of this combination on the toxicity of the individual members. In view of the poor water solubility of the Anthallan base, a 10% aqueous supension was prepared for oral administration. At 3.0 Gm./Kg. in albino mice there was no toxicity and only slight catharsis. This represents the upper practical volume for oral administration to mice. As an alternate approach to the problem, the $LD_{50}$ for aminophylline in mice was determined and the test repeated using a solution-suspension of aminophylline and Anthallan in equal quantities. The results are summarized in Table III. Statistical evaluation by the method of Litchfield and Wilcoxon (8) indicates that (1) the data are homogeneous; (2) the curves of the two assays are parallel, and (3) the difference in toxicity is not significant.

In addition to the acute toxicity, chronic toxicity studies in rats were conducted, using male and female mates from three litters distributed to provide maximum group uniformity. One control group and two test groups of five rats each were established. The first test group received 0.132% aminophylline in the diet. This amount was selected to provide approximately 20.0 mg./rat/day, assuming a 15.0-Gm. daily food consumption. At initial body weights this amount would exceed one-half the oral lethal dose as determined in mice.

The second test group received a diet containing 0.132% each of aminophylline and Anthallan. After fifty-two days there was (1) no mortality during the test; (2) no significant difference in weight, either initial or terminal; (3) no alteration in normal growth curve; (4) no significant difference in the percentage of weight gained. It is interesting that the group receiving aminophylline and Anthallan combined ate significantly more than the controls ($p = 0.01$).

## DISCUSSION AND SUMMARY

Anthallan is a water-insoluble base capable of forming soluble salts such as the hydrochloride and gluconate. Both the base and the salts are of a relatively low order of toxicity when administered to mice, cats, or dogs parenterally. Under these conditions both dogs and cats show defecation and cats show an excitatory state. When administered orally to cats or dogs, defecation is the only visible effect induced by the base, but in addition the hydrochloride induces nausea and vomiting.

Pharmacologically, Anthallan and Anthallan salts are relatively inert, the most consistent observation being a mild vasodepressor action which has been shown to be nonspecific in nature. Prolonged feeding of the base to mice, rats, guinea pigs, and cats produces no grossly observable effects, and histopathological examinations at autopsy were negative.

When tested by the vasodepressor response to histamine in cats and dogs, Anthallan exhibits only a mild antihistaminic effect; thus, it is capable of eliminating the depressor effect of doses at or slightly above the threshold but not the effect from relatively large doses of histamine.

It has been shown that following oral administration Anthallan is rapidly absorbed and is excreted at moderate rates via the urine. A large portion of the excretion is in the form of conjugation products which require hydrolysis before they are detectable.

Chemical and biological studies indicate that a mixture of Anthallan with aminophylline remains as a discrete mixture under physiological conditions and that this mixture does not affect the acute or chronic toxicity of the individual components.

In clinical practice large doses of Anthallan are used over a prolonged period of time, and there is a considerable period of maintained effect following withdrawal of the drug. That the clinical results thus achieved may be independent of the mild antihistaminic action of Anthallan is suggested by recent work of Castillo (9) indicating that on tracheal chains the very potent antihistaminics do not counteract allergic contractions of the trachea. The results of the investigations summarized in this report indicate that prolonged and relatively large doses may be accomplished without toxic manifestations.

TABLE III.—ACUTE ORAL TOXICITY FOR MICE OF AMINOPHYLLINE AND AMINOPHYLLINE ACCOMPANIED BY AN EQUAL AMOUNT OF ANTHALLAN, MALE AND FEMALE MICE

| Compound | Oral Dose, Mg./Kg.[a] | | | | | $LD_{50}$ | Error Factor | Limits of Error |
|---|---|---|---|---|---|---|---|---|
| | 300 | 400 | 450 | 500 | 600 | | | |
| Aminophylline | 0/6 | 3/10 | 6/10 | 8/10 | ... | 435 | 1.082 | 402–471 |
| Aminophylline plus Anthallan | ... | 2/10 | ... | 4/10 | 8/10 | 500 | 1.118 | 447–558 |

[a] Values under each dose represent number of mice dead/number of mice tested. Exploratory doses beyond the indicated range are omitted.

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# Determination of *d*-Tubocurarine Chloride*,†

By DANIEL KLEIN and SAMUEL M. GORDON

**Rapid and convenient procedures for the determination of *d*-tubocurarine chloride in aqueous solution based on direct ultraviolet spectrophotometric determination and on colorimetric determination of the Reineckate complex are reported. These procedures give results agreeing closely with calculated values.**

PHYSICAL or chemical methods for the assay of *d*-tubocurarine chloride do not appear in the available literature (1). It is presently assayed by a biological method (2–4). While this head drop method is precise, its application is limited to laboratories with facilities and personnel for bioassay. Furthermore, the method is time-consuming and is not suitable for routine control purposes. Since *d*-tubocurarine chloride is a very potent drug and one which possesses great potentiality for harm if improperly dispensed or used, the advantages of simple and readily performed assay procedures for analytical control purposes are apparent. The recently published standards of the Council on Pharmacy and Chemistry (1) suggest that the optical rotation may be used to determine *d*-tubocurarine chloride. Relatively concentrated solutions may be required for reliable results, and optical rotation alone could obviously not be used for identification and assay.

The availability of the drug in a state which is very close to the upper limits of purity[1] in the requirements of the Council on Pharmacy and Chemistry (1) provides the basis for the methods reported from this laboratory. Three methods, which are readily performed and offer a high degree of accuracy, are described. These are based on (*a*) optical rotation, already alluded to, (*b*) spectrophotometric absorption in the ultraviolet region, (*c*) spectrophotometric absorption of the Reineckate in the visible region. A study of the ultraviolet absorption curve of the salt in aqueous solution showed an absorption maximum at 280–281 mμ. Thus direct extinction measurement at 280.5 mμ was found to be an exact analytical method. As a quaternary ammonium salt *d*-tubocurarine chloride forms an insoluble Reineckate (1). The *d*-tubocurarine Reineckate dissolved in alcohol is measured colorimetrically at 525 mμ. This third procedure is similar to the method for homatropine methyl bromide (5). The operations are simplified by conducting the entire procedure in a centrifuge tube.[2]

## EXPERIMENTAL

**Apparatus.**—Beckman Spectrophotometer, Model DU with light sources for visible and ultraviolet ranges.[3] Matched 1-cm. cuvettes, glass, and quartz. Centrifuge tubes, 15 ml. graduated, calibrated at 10.0 ml. Rubber caps, to fit mouth of centrifuge tubes, made by cutting off the solid plug of a rubber stopper, pure gum, sleeve type No. 11 (The West Co., Phoenixville, Pa.). Polarimeter: a Schmidt and Haensch instrument with a scale permitting readings to the third decimal place.

**Reagents.**—*d-Tubocurarine Chloride.*[4]—A colorless, crystalline powder was employed, the comparison of which, with the recently announced standards, is outlined in Table I (1).

TABLE I

| | Council Standards | Found |
|---|---|---|
| Specific rotation $[\alpha]_D^{25}$, anhydrous | +208° to 217°[a] | +215° |
| Specific rotation $[\alpha]_D^{25}$, as pentahydrate | ..... | +190° |
| Chloride, as anhydrous salt | 9.5% to 10.2% | 10.0% |
| Chloroform extractable residue, on anhydrous basis | Not more than 3% | 0.9%[b]<br>0.2% |
| Bioassay, $HD_{50}$ as pentahydrate | 0.15 mg./Kg. | 0.145 mg./Kg. |

[a] According to (1) the most probable value for specific rotation of pure anhydrous *d*-tubocurarine chloride is +215°.
[b] 0.9% obtained using "Silicone" as lubricant for the separatory funnel; 0.2% obtained using phosphoric acid, U. S. P. as lubricant.

*d-Tubocurarine Chloride Pentahydrate Standard Solution.*—Dry 400 mg. to constant weight at 100° (four hours), and transfer to a 250-ml. volumetric

* Received Jan. 10, 1949, from The Laboratories of Endo Products Inc., Richmond Hill 18, N. Y.
† Dr. Paul Fehder and Miss Adele Moore carried out some of the determinations reported herein.
[1] *d*-Tubocurarine chloride in text under Reagents.
[2] This simplified procedure is now also being used in this laboratory for the determination of homatropine methyl bromide.
[3] Other instruments are also suitable.
[4] Obtained from R. C. Gill, Palo Alto, Calif., to whom we are indebted for the $HD_{50}$ value of 0.145 mg./Kg. and for other information on the properties of *d*-tubocurarine chloride.

flask containing 1.25 Gm. chlorobutanol,[8] and make to volume with water. Express as the pentahydrate by multiplying the anhydrous weight by 1.13, to yield a solution in the range of 1.8 mg. per ml. Store in an amber bottle in the refrigerator.

*Reinecke Salt (Ammonium Reineckate, Eastman Kodak) 2 Per Cent Solution.*—Prepare fresh on day of use by dissolving in water with continuous mixing for fifteen to twenty minutes, using a mechanical stirrer. Before using, centrifuge and decant the clear supernatant solution into a flask.

*Sulfuric acid 20% solution.*—57 ml. of concentrated acid to 500 ml. with water.

*Ethyl alcohol.*—Absolute, with 0.5% benzene (S. D., No. 2B absolute).

**Ultraviolet Spectrophotometric Measurements.**—The absorption spectrum was determined by making extinction measurements from 240–300 mμ of an aqueous solution of *d*-tubocurarine chloride pentahydrate containing 4.2 mg. per 100 ml. An absorption peak was obtained at 280–281 mμ with a molecular extinction coefficient of 8000.

To determine the applicability of the Beer-Lambert law, measurements were made at 280.5 mμ of a series of solutions containing 3 to 11 mg. of the pentahydrate per 100 ml. Table II records the results obtained. The average value for $E^{1\%}_{1\text{ cm. }280.5\text{ m}\mu}$ of 101.6 (range 101–102) shows that the Beer-Lambert law holds for *d*-tubocurarine chloride at 280.5 mμ.

TABLE II.—RELATIONSHIP BETWEEN CONCENTRATION OF *d*-TUBOCURARINE CHLORIDE AND EXTINCTION

| *d*-Tubocurarine Chloride Pentahydrate, Mg./100 Ml. | $E_{1\text{ cm. }280.5\text{ m}\mu}$ | $E^{1\%}_{1\text{ cm. }280.5\text{ m}\mu}$ |
|---|---|---|
| 3.00 | 0.307 | 102 |
| 3.60 | 0.368 | 102 |
| 4.20 | 0.424 | 101 |
| 4.43 | 0.451 | 102 |
| 4.50 | 0.456 | 101 |
| 4.80 | 0.486 | 101 |
| 6.00 | 0.611 | 102 |
| 6.00 | 0.612 | 102 |
| 8.86 | 0.900 | 102 |
| 11.1 | 1.122 | 101 |
| | Average | 101.6 |

**Preparation and Measurement as Reineckate.**—A series of solutions containing 4 to 21 mg. of *d*-tubocurarine chloride pentahydrate per 10 ml. of water was pipetted into 15-cc. graduated centrifuge tubes. One milliliter of 20% sulfuric acid followed by 3 ml. of 2% Reinecke salt solution were added. The tubes were stoppered with rubber caps and mixed by inversion. After standing for one hour to allow for complete precipitation of the pink Reineckate, the tubes were centrifuged for ten minutes. The caps were removed, the supernatant liquid was decanted and excess liquid was drawn off by pressing the mouth of the tube on toweling or filter paper. The precipitate and walls of the tube were washed twice with 1-ml. portions of water, each time centrifuging for two minutes, decanting and removing excess liquid on filter paper.

Absolute alcohol was then added so that the volume measured 10 ml. after complete solution of the Reineckate. This step was accomplished by bringing volume to 10.1 ml., covering the tube with a rubber cap, followed by vigorous shaking. The pink solutions obtained were usually slightly turbid. Centrifugation for one minute cleared the solutions.

Alcoholic solutions prepared in this way showed an absorption peak at 525 mμ. The color was stable for at least twenty-four hours. Applicability of the Beer-Lambert law to alcoholic solutions of the Reineckate of *d*-tubocurarine chloride are illustrated in Table III. The deviation, ±3%, from the average

TABLE III.—RELATIONSHIP BETWEEN CONCENTRATION OF *d*-TUBOCURARINE REINECKATE AND EXTINCTION

| Mg./10 Ml. Alcohol[a] | $E_{1\text{ cm. }525\text{ m}\mu}$ | $E^{1\%}_{1\text{ cm. }525\text{ m}\mu}$ |
|---|---|---|
| 4.36 | 0.133 | 3.05 |
| 4.36 | 0.134 | 3.07 |
| 5.45 | 0.166 | 3.05 |
| 5.45 | 0.162 | 2.97 |
| 6.54 | 0.193 | 2.95 |
| 8.72 | 0.269 | 3.09 |
| 10.9 | 0.335 | 3.07 |
| 10.9 | 0.337 | 3.09 |
| 10.9 | 0.326 | 2.99 |
| 10.9 | 0.329 | 3.02 |
| 13.1 | 0.395 | 3.02 |
| 17.4 | 0.523 | 3.00 |
| 21.8 | 0.650 | 2.98 |
| 21.8 | 0.662 | 3.04 |
| | Average | 3.03 ± 0.08 |

[a] Concentration of *d*-tubocurarine Reineckate expressed as free *d*-tubocurarine chloride pentahydrate.

$E^{1\%}_{1\text{ cm. }525\text{ m}\mu}$ of 3.03 compares with a deviation of less than 1% from the average $E^{1\%}_{1\text{ cm. }208.5\text{ m}\mu}$ of 101.6 (Table II) by the ultraviolet spectrophotometric method. The greater sensitivity of the ultraviolet spectrophotometric method was not unexpected, since it entails a simple dilution technique, whereas the colorimetric procedure requires a number of steps.

**Method for Solutions of *d*-Tubocurarine Chloride**

**Optical Rotation Method.**—Determine optical rotation of solution at 25° using either a 1 or 2-dm. tube. Calculate concentration from the following formula:

$$\text{Mg. } d\text{-tubocurarine chloride pentahydrate per ml.} = \frac{1000a}{+190 \times l}$$

$a$ = observed rotation at 25°

$+190 = [\alpha]^{25}_{D}$ for *d*-tubocurarine chloride pentahydrate

$l$ = tube length in dm.

Since multiple dose vials of *d*-tubocurarine chloride are prepared with 0.5% chlorobutanol as preservative, the latter was added to the standard solution.

Example: $a = +1.097°$
$l = 2$ dm.

$$\frac{1000 \times 1.097}{190 \times 2} = 2.9 \text{ mg. per ml.}$$

**Ultraviolet Spectrophotometric Method.**—From the value obtained by the optical rotation method make an appropriate dilution for a final concentration of 3 to 10 mg. per 100 ml. Using 1-cm. matched quartz cuvettes, determine optical density of this diluted solution at 280.5 mμ. From the value for $E^{1\%}_{1 \text{ cm. } 280.5 \text{ m}\mu}$ derived as described under Ultraviolet Spectrophotometric Measurements, calculate concentration as follows:

$$\text{Mg. } d\text{-tubocurarine chloride pentahydrate per ml.} = \frac{E_{1 \text{ cm. } 280.5 \text{ m}\mu} \text{ of diluted solution} \times \text{vol. in ml. of diluted solution} \times 10}{E^{1\%}_{1 \text{ cm. } 280.5 \text{ m}\mu} \times \text{ml. of original solution taken}}$$

Example: 3 ml. diluted to 500 ml.

$$E_{1 \text{ cm. } 280.5 \text{ m}\mu} = 0.488$$
$$E^{1\%}_{1 \text{ cm. } 280.5 \text{ m}\mu} = 101.6$$
$$\frac{0.488 \times 500 \times 10}{101.6 \times 3} = 8.00 \text{ mg. per ml.}$$

**Colorimetric Method.**—Pipette a volume of solution estimated to contain between 5 and 10 mg. of $d$-tubocurarine chloride pentahydrate into a pair of 15-ml. graduated centrifuge tubes. For more precise results, the Standard Solution should also be carried through at the same time. Measure a volume of Standard Solution into another pair of centrifuge tubes, to contain a concentration of the pentahydrate close to the expected value for the unknown. Bring volume in all tubes to 10 ml. and continue as described under Preparation and Measurement as Reineckate. Calculate concentration as follows with average $E$ values of standard and unknown solutions.

$$\text{Mg. } d\text{-tubocurarine chloride pentahydrate per ml.} = \frac{E_{1 \text{ cm. } 525 \text{ m}\mu} \text{ of unknown solution} \times \text{mg. of standard solution}}{E_{1 \text{ cm. } 525 \text{ m}\mu} \text{ of standard solution} \times \text{ml. of unknown solution taken}}$$

Example: 6.00 mg. of standard solution, $E_{1 \text{ cm. } 525 \text{ m}\mu} = 0.181$

2 ml. unknown solution, $E_{1 \text{ cm. } 525 \text{ m}\mu} = 0.226$

$$\frac{0.226 \times 6}{0.181 \times 2} = 3.74 \text{ mg. per ml.}$$

The three methods of assay have been applied to $d$-tubocurarine chloride solutions of known and unknown content.

Table IV deals with analytical results obtained on solutions in bulk, and in sterile containers, labeled at 3 mg. and 15 mg. $d$-tubocurarine chloride per ml. Similar results illustrated in Table V were obtained on solutions prepared for this study by Dr. A. E. Sidwell, Jr., of the A. M. A. Chemical Laboratory. The optical rotation method indicated the approximate concentration. Values by the ultraviolet spectrophotometric and the colorimetric Reineckate procedures showed very close agreement, with a deviation of zero to 1% from the average values. In regard to the concentrations found in the unknown solutions (Table V), these were from 0 to 3% higher than the labeled content. Thus the combination of visible and ultraviolet spectrophotometric methods for exact assay along with the optical rotation method for added identification furnish an appropriate means to determine $d$-tubocurarine chloride in solution.

TABLE IV.—DETERMINATION OF $d$-TUBOCURARINE CHLORIDE CONTENT IN AQUEOUS SOLUTIONS OF KNOWN CONCENTRATION

| Code[a] | Description | Mg. $d$-Tubocurarine Chloride Pentahydrate per Ml. Found by Method— Optical Rotation (A) | Spectrophotometric (B) | Colorimetric (C) | Average Methods (B) and (C) | Deviation from Average % | % of Labeled Amount. Average of (B) and (C) |
|---|---|---|---|---|---|---|---|
| | *Labeled 3 Mg. per Ml.* | | | | | | |
| 770 | Vial, 10 cc. | 2.9 | 3.02 | 3.03 | 3.03 | 0.3 | 101 |
| 770 | After 2 hrs. aeration | 3.0 | 3.05 | 3.05 | 3.05 | 0.0 | 102 |
| 770 | One month at 37° | 2.9 | 3.09 | 3.10 | 3.10 | 0.3 | 103 |
| 810 | Bulk solution as prepared | 3.2 | 3.44 | ... | ... | ... | ... |
| 810 | Adjusted | ... | 3.10 | 3.09 | 3.10 | 0.3 | 103 |
| | *Labeled 15 Mg. per Ml.* | | | | | | |
| 887 | Bulk solution, as prepared | 16.8 | 17.3 | ... | ... | ... | ... |
| 887 | Adjusted | ... | 15.6 | ... | ... | ... | ... |
| 887 | Ampul, 1 cc. | 15.1 | 15.5 | 15.4 | 15.45 | 0.3 | 103 |

[a] All these solutions were prepared from $d$-tubocurarine chloride pentahydrate supplied by Richard C. Gill, Palo Alto Calif., and were water-white.

## SUMMARY

1. A multiple chemical assay procedure has been developed for $d$-tubocurarine chloride in dilute solution based on three different properties of the substance.

TABLE V.—DETERMINATION OF *d*-TUBOCURARINE CHLORIDE CONTENT IN AQUEOUS SOLUTIONS OF UNKNOWN CONCENTRATION

| Code | As Prepared,[a] Mg. per Ml. | Mg. *d*-Tubocurarine Chloride Pentahydrate per Ml. Found by Method— Optical Rotation (A) | Spectrophotometric (B) | Colorimetric (C) | Average Methods (B) and (C) | Deviation from Average, % | Difference of Average from Stated Content, % |
|---|---|---|---|---|---|---|---|
| S 921 | 3.25 | 3.8 | 3.29 | 3.22 | 3.25 | 1.3 | 0.0 |
| S 924-1 | 8.13 | 8.1 | 8.30 | 8.43 | 8.36 | 0.8 | 2.83 |
| S 924-5 | 9.16 | 8.8 | 9.22 | 9.30 | 9.26 | 0.4 | 0.09 |
| S 924-7[b] | 10.92 | 11.0 | 11.2 | 11.1 | 11.15 | 0.5 | 2.11 |
| S 924-8 | 10.02 | 9.5 | 10.0 | 10.1 | 10.05 | 0.5 | 0.30 |
| S 925 | 3.67 | 3.4 | 3.77 | 3.81 | 3.79 | 0.5 | 3.27 |
| S 927[b] | 4.37 | 4.3 | 4.42 | 4.44 | 4.43 | 0.2 | 1.37 |
| S 928 | 4.02 | 3.6 | 4.10 | 4.14 | 4.12 | 0.5 | 2.49 |

[a] These solutions were furnished by Dr. A. E. Sidwell, Jr., of the American Medical Association Chemical Laboratory and sent to us under code.
[b] S 924-7 and S 927 had a yellow and slight yellow color; the others were water-white.

2. Measurement of optical rotation gives a roughly quantitative value; direct determination of the extinction at 280.5 mμ and photometric determination of the pink Reineckate in alcoholic solution at 525 mμ yield values which agree with each other and with the known concentrations.

3. Application of the methods to solutions demonstrates that the combination of all three procedures may be utilized to identify and determine *d*-tubocurarine chloride in solution.

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# A Practical Method for the Biological Assay of Heparin*

By M. N. LEWIS and FRANCESCO De MARIA

**A method is described whereby citrated beef plasma is recalcified with calcium chloride in the presence of increasing amounts of heparin followed by an evaluation of the clot formation. Results of numerous assays are tabulated and illustrated. The procedure described has been found useful for the routine assay of the anticoagulant activity of heparin.**

THE VARIOUS METHODS used for the determination of heparin potency are based on the inhibition of clot formation in whole blood (1–5) or in the plasma (6–8). The method most widely used is that of Reinert and Winterstein (7) (decalcified beef plasma and added calcium chloride) which has been successfully modified and refined by Foster (9), and Kuizenga, *et al.* (10). More recently, Mangieri (11) suggested modifications to Foster's method.

The rapidity and high degree of reproducibility of the technique developed in our laboratory, although they do not involve new principles, have prompted this publication.

## MATERIALS AND METHODS

**Plasma.**—Beef blood, collected directly from the slaughtered animal in large, wide-mouthed vessels containing 100 cc. of a 4.0% citrate solution per liter of blood, is centrifuged, as soon as is feasible, for two hours, and the clear plasma drawn off by suction. This plasma is usable for four or five days if kept at 3°; or it may be slow-frozen and kept for months, in which case the amount required for a day's assay is thawed in a 37° water bath. Straining through glass wool removes any clot which may appear in this stock plasma.

**Recalcification.**—The citrated plasma is recalcified with calcium chloride, the optimum amount of which must be determined each day of assay. Table I shows the reagents and volumes used in this titration. The reagents are dispensed from 5 cc. microburettes calibrated to 0.01 cc., into a series of 75- ×

* Received May 19, 1949, from the Warner Institute for Therapeutic Research, New York City.

TABLE I.—REAGENTS AND VOLUMES USED IN THE DETERMINATION OF THE AMOUNT OF $CaCl_2$ TO BE USED IN HEPARIN ASSAY[a]

| Test Tube No. | Saline (0.9%) Cc. | Heparin (5 γ/Cc.), Cc. | $CaCl_2$ (5 Mg./Cc.) Mg. | $CaCl_2$ (5 Mg./Cc.) Cc. | Plasma, Cc. |
|---|---|---|---|---|---|
| 1 | 0.55 | 0.40 | 0.25 | 0.05 | 1.00 |
| 2 | 0.50 | 0.40 | 0.50 | 0.10 | 1.00 |
| 3 | 0.45 | 0.40 | 0.75 | 0.15 | 1.00 |
| 4 | 0.40 | 0.40 | 1.00 | 0.20 | 1.00 |
| 5 | 0.35 | 0.40 | 1.25 | 0.25 | 1.00 |
| 6 | 0.30 | 0.40 | 1.50 | 0.30 | 1.00 |
| 7 | 0.25 | 0.40 | 1.75 | 0.35 | 1.00 |
| 8 | 0.20 | 0.40 | 2.00 | 0.40 | 1.00 |
| 9 | 0.15 | 0.40 | 2.25 | 0.45 | 1.00 |
| 10 | 0.10 | 0.40 | 2.50 | 0.50 | 1.00 |

[a] In the event that the 50% clot falls between two tubes, and is therefore estimated, 3 to 4 $CaCl_2$ concentrations within the limits found here should be used in trial heparin-assay rows, and that concentration of $CaCl_2$ selected which produces the best curve.

10-mm. test tubes which are plugged with rubber stoppers. (We have found these as successful as the paraffined corks mentioned by previous authors.) *All* glassware should be chemically clean. The order in which the reagents are added—saline, heparin, $CaCl_2$, plasma—*must* be observed. (The succession: plasma, heparin, saline, $CaCl_2$ gave less consistent results. This might be in relation to our observation that the longer the contact between heparin and plasma, the less consistent are the gradations in the clotting.)

After two hours' incubation at 37°, the percentage of clot formation is evaluated for each tube and the amount (in mg.) of $CaCl_2$ *which produces a 50% clot formation* is determined. A solution containing this amount of $CaCl_2$ per 0.2 cc. is prepared for the assay.

**Assay.**—The reagents and volumes used in the assay are shown in Table II. Upon addition of plasma to each tube, the tube is stoppered and inverted several times to mix; each rack of tubes is incubated as it is finished. Although tubes containing saline and heparin may be allowed to stand for some time, it was found that after the addition of $CaCl_2$, for consistent results the plasma should be added immediately. We have no rational explanation to offer for this finding. The assay tubes are incubated at 37° for three hours and then read. Two series of standard heparin tubes are done each day, one at the beginning and one at the end of the assay, and the readings pooled.

**Reading.**—The clot formation in each tube is determined by separating the clot from the unclotted plasma and evaluating (1) the amount of fluid covering the cork of the inverted tube while the clot clings to the bottom part of the test tube, or (2) the size of the floating clot, or both. Thus, complete clotting = 100% = no fluid; three-quarters clotting = 75% = one-quarter fluid; no clotting = 0% = total fluid; etc. Since, once disturbed, clot formations change rapidly, each tube should be read immediately upon the first separation of clotted from unclotted plasma. Rapping sharply against the bottom half of the inverted test tube with a ruler or glass rod covered with heavy rubber tubing will facilitate the desired separation of clot and fluid.

The percentage of clotting, as measured, plotted against the heparin concentration, yields a curve of characteristic form (Fig. 1). The equivalent

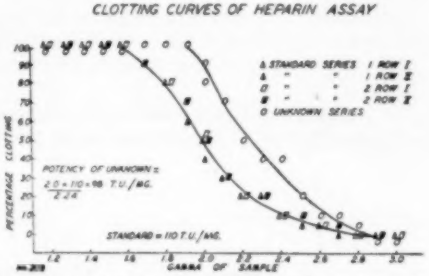
CLOTTING CURVES OF HEPARIN ASSAY
PERCENTAGE CLOTTING
CAGGA OF SAMPLE
STANDARD SERIES I ROW I
STANDARD SERIES I ROW II
STANDARD SERIES 2 ROW I
STANDARD SERIES 2 ROW II
UNKNOWN SERIES
POTENCY OF UNKNOWN =
STANDARD = 110 T.U./MG.

Fig. 1.—Clotting curve of an unknown sample ("unknown series") compared to that of 2 standard samples (1 and 2), determined on 2 rows (*I* and *II*) for each.

amounts of standard and unknown are read from the curve at the zone of greatest accuracy; i.e., 50% clotting. These figures applied in the following formula give the potency of the unknown samples:

$$\frac{\gamma \text{ of standard} \times \text{units/mg. of standard}}{\gamma \text{ of unknown}} = \text{units/mg. of unknown}$$

TABLE II.—CONCENTRATIONS AND VOLUME OF REAGENTS USED IN HEPARIN ASSAY (1 RACK)

| | Row I | | | | Row II | | | |
|---|---|---|---|---|---|---|---|---|
| Test Tube No. | Saline (0.9%), Cc. | Heparin[a] (5 γ/Cc.), Cc. | $CaCl_2$,[b] Cc. | Plasma, Cc. | Saline (0.9%), Cc. | Heparin[a] (5 γ/Cc.), Cc. | $CaCl_2$,[b] Cc. | Plasma, Cc. |
| 1 | 0.56 | 0.24 | 0.20 | 1.00 | 0.54 | 0.26 | 0.20 | 1.00 |
| 2 | 0.52 | 0.28 | 0.20 | 1.00 | 0.50 | 0.30 | 0.20 | 1.00 |
| 3 | 0.48 | 0.32 | 0.20 | 1.00 | 0.46 | 0.34 | 0.20 | 1.00 |
| 4 | 0.44 | 0.36 | 0.20 | 1.00 | 0.42 | 0.38 | 0.20 | 1.00 |
| 5[c] | 0.40 | 0.40 | 0.20 | 1.00 | 0.40 | 0.40 | 0.20 | 1.00 |
| 6 | 0.36 | 0.44 | 0.20 | 1.00 | 0.38 | 0.42 | 0.20 | 1.00 |
| 7 | 0.32 | 0.48 | 0.20 | 1.00 | 0.34 | 0.46 | 0.20 | 1.00 |
| 8 | 0.28 | 0.52 | 0.20 | 1.00 | 0.30 | 0.50 | 0.20 | 1.00 |
| 9 | 0.24 | 0.56 | 0.20 | 1.00 | 0.26 | 0.54 | 0.20 | 1.00 |
| 10 | 0.20 | 0.60 | 0.20 | 1.00 | 0.22 | 0.58 | 0.20 | 1.00 |

[a] This concentration is used for samples with potencies of 50–120 Toronto u./mg. Other samples require adjustments so as to have approximately 0.5 Toronto u./cc.
[b] Concentration as determined in Table I.
[c] Zone of the 50% clot with samples of 110 u./mg. potency.

## DISCUSSION

This method has been used for routine daily assay of heparin samples in this laboratory for over two and a half years with a high degree of reproducibility among 6 different technicians. It has the advantage of permitting a quantitative evaluation of the actual amount of clotting. As the heparin concentrations are selected so as to produce an inclusive curve, from 100% to 0% clotting, the most characteristic zone of clotting (50%) can be used in the computations. Two workers reading the same series of tubes will have practically identical curves.

It has been our experience that the less $CaCl_2$ and heparin used in the clotting system, the greater the sensitivity of the method.[1] It is considered that the relatively small concentrations of these reagents, and the small heparin increment (0.1 γ per test tube) are responsible for our favorable results

[1] For this reason blood "overcitrated" during collection is not suitable for use.


## SUMMARY

A method for the routine assay of the anticoagulant activity of heparin samples is presented.

Citrated beef plasma is recalcified with $CaCl_2$ in the presence of increasing amounts of heparin, and the percentage of the clot formation evaluated. Unknown and standard samples are compared at the point of 50 per cent clotting.


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# A Contribution to the Chemistry and Toxicology of the Root of *Phytolacca americana*, L.*

By Z. F. AHMED, C. J. ZUFALL, and G. L. JENKINS


**The extraction of the dried powdered root according to the successive solvent extraction method, the general lead method, and the Stas-Otto method did not establish the presence of an alkaloid, but did establish the presence of the following substances: starch, gum, hemicellulose, sucrose, oxalates, nitrates, steroid materials, and at least one water-soluble saponin. In further search for an alkaloid, the dried powdered root, as well as the expressed juice of the fresh root, was subjected to extensive investigation which embodies various techniques, namely: solvent extraction, precipitation, adsorption, and distillation—with negative results. By pharmacological studies, the toxicity of the root, by elimination, was confined to certain extractives and ultimately led to the acidic steroid sapotoxin, m. p. 212°, the physiological activity of which was correlated in all respects to the reported physiological activity of the root and its crude extract. This crystalline toxic principle was subjected to physical, chemical, and pharmacological examination.**


A CONCISE RECORD of the results obtained by previous workers on poke root (*Phytolacca americana* Linné) appeared in an earlier publication by Jenkins (1).

The conflicting reports about the existence of an alkaloid in the root, in spite of the fact that a substantial evidence as to its presence is completely lacking, and the unavailability of specific knowledge concerning the physiologically active constituent or constituents present warrant the work undertaken.

The experiments carried out in search for an alkaloid evidently demonstrated its absence, and a pharmacological lead to trace the active constituent of the drug was a necessity.

This lead, by elimination, confined the toxicity of the drug to certain extractives and ultimately led to an acidic steroid saponin, m. p. 212°, whose physiological activity was correlated in all respects to the reported physiological activity of the drug and its crude extracts.


* Received April 30, 1949, from the laboratories of the Pharmacognosy Department of Purdue University, School of Pharmacy.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.

## EXPERIMENTAL

### Determination of Characteristics

Using U. S. P. XIII methods, the following characteristics were determined for the powered poke root, N. F., supplied by S. B. Penick and Co.

Moisture, 9.05%; total ash, 10.31%; acid-insoluble ash, 2.74%.

Dilute alcohol extractives, 15.43%; alcohol-soluble extractive, 3.13%; benzin-soluble extractive, 1.01%; ether-soluble extractive, 0.71%; nonvolatile ether-soluble extractive 0.62%; volatile ether-soluble extractive, 0.09%; and water-soluble extractive, 16.63%.

### A Search for an Alkaloid

The extraction of the dried powdered root according to the successive solvent extraction method (2), to the general lead method (3), and to the Stas-Otto method (4) did not establish the presence of any alkaloid but established the presence of the following substances: starch (5–7), gum (5–8), oxalates (6, 7), hemicellulose (6), steroid materials (6), and at least one water-soluble saponin.

For further search for an alkaloid the dried powdered root, as well as the expressed juice of the fresh root, was subjected to extensive investigation which embodies various techniques, such as solvent extraction, precipitation, adsorption, and distillation.

The results obtained did not establish the presence of an alkaloid.

## DISCUSSION

It was noticed that all the residues in which the presence of an alkaloid was a possibility rather than a probability, did not have any significant basic properties. Other residues which might probably contain an alkaloid did not show any evidence of its presence.

Besides Preston's claim (9) of isolating a crystalline alkaloidal substance (which was repeated in previous work (6) and in this work without confirming his results) the only evidence of the presence of an alkaloid was the fact that the plant, in the form of its crude extracts or extractive residues, gave amorphous precipitates with some of the so-called general alkaloidal reagents (6, 8, 10).

The most sensitive of these reagents, as was reported in the literature and as was confirmed in the present work, were phosphotungstic acid, phosphomolybdic acid, and tannic acid test solutions.

Most of the residues which gave precipitates with these reagents responded to the Molisch test. Moreover, at least one steroid water-soluble saponin, which gave amorphous precipitates with these reagents, was isolated. Whether this precipitation was due only to the saponin or to the saponin and the variable impurities, carbohydrates or otherwise, held so tightly to its surface, did not change the fact that the precipitates themselves were alkaloid-free.

This evidence was further substantiated by the fact that these residues, which were supposed to contain the alkaloid, on further purification failed to precipitate with the reagents, instead of becoming more sensitive.

It was also found that in those cases where nitrogen was detected in the purified residue, nitrate ion was invariably present. Prolonged dialysis failed to remove the last traces of nitrogen from the water-soluble saponin. The exceptionally high concentration of potassium nitrate in the plant (5, 6, 8) made this contamination easy.

### Tracing of the Toxic Constituent of the Drug

Normal saline suspensions of each of the following dried extracts of poke root were prepared so that each milliliter of the suspension represented one gram of the dried powdered drug: ether extract, chloroform extract, Prollius fluid extract, alcohol extract, and water extract.

One cubic centimeter of each of these suspensions was injected intraperitoneally into rats (average weight 250–300 Gm.).

All the animals survived in apparently normal condition except the animal injected with the alcoholic extract suspension. This animal died after ten hours with the following symptoms:

Following the injection the animal showed catharsis and uresis. During the following two hours the animal was extremely sensitive to the least outside disturbance. This condition then faded away to drowsiness which continued and increased with the progress of time. Later, breathing became labored. Near the death of the animal the dyspnea was very marked and the animal started to lose sensation in its tail. This paralytic condition ascended, resulting in complete loss of function of the lower limbs of the animal, failure of respiration, and death.

This preliminary experiment indicated that the toxic constituent or constituents of the drug were present, perhaps in the highest concentration, in the alcoholic extract of the drug. The further chemical investigation then followed this lead.

### A Preliminary Experiment

One kilogram of the dried powdered drug was completely defatted by petroleum ether (b. p. 30–60°) in a continuous extraction apparatus. The marc was dried in air until all the solvent was removed and then exhausted by continuous extraction with 95% alcohol. Most of the alcohol was removed from the clear, reddish brown extract by distillation. The dark, reddish brown, concentrated extract which amounted to 200 cc. deposited on cooling a white, amorphous, fluffy substance and a great number of acicular crystals of potassium nitrate.

The alcoholic extract was filtered and the residue (*A*) was set aside for further examination.

The filtrate was then thrown into a large volume of water (about 2 liters). The whitish, voluminous, fluffy precipitate (*B*) took about ten hours for complete sedimentation leaving an orange aqueous supernatant liquid which was decanted with difficulty since the precipitate was easily disturbed. The precipitate was separated without difficulty from the remainder of the supernatant fluid by centrifugation, redissolved in alcohol, and thrown into water. This process was repeated until the aqueous solution was completely free from extractive matter as shown by absence of any color.

The combined aqueous solutions were concentrated by evaporation on a water bath to a small bulk, filtered, and evaporation was continued to dryness on the water bath. The sticky, reddish

brown residue was redissolved in a little water and precipitated with absolute alcohol-ether mixture. This process was repeated twice and the precipitate (*C*) was dried and kept under vacuum over calcium chloride for further examination.

The precipitate (*B*) gave on drying under vacuum over calcium chloride about 6.5 Gm. of a brownish resinous mass which was soluble in solutions of alkalies, namely potassium and sodium hydroxides, and insoluble in dilute acids, namely hydrochloric acid, sulfuric acid, and nitric acid. It was undoubtedly the acid resin of Donelly (5), Pape (7), and others.

Thirty milligrams of this residue suspended in normal saline and injected intraperitoneally into a 300-Gm. rat, killed the animal in two hours with the same general symptoms described previously under the alcoholic extract.

The toxic residue was further purified by dissolving it in a 5% solution of potassium hydroxide solution, filtering, and acidifying the filtrate with 10% sulfuric acid. The substance was deposited as a gelatinous, whitish mass which was separated by centrifugation. The deposit was dissolved in absolute alcohol and precipitated by the addition of chloroform, then dissolved in alcohol and precipitated by the addition of ether. The chloroformic and ethereal liquors (*W*) were combined and kept for further examination. Finally the precipitate was dissolved in alcohol and evaporated to dryness. The scaly, grayish yellow, dried residue amounted to about 3.0 Gm. and had a strong sternutatory effect. This was most probably the proximate principle of Trimble (11).

The substance was then transferred to an Erlenmeyer flask and refluxed with absolute ether for one hour. The deep yellow ethereal extract was filtered off and the residue was again refluxed with a fresh portion of absolute ether for another hour. This process was repeated once more until the ethereal solution was completely colorless. The combined ethereal extracts (*W*2) were kept for further examination.

The whitish, fluffy substance remaining after refluxing with ether was air dried by the use of suction in a Büchner funnel and dissolved in alcohol. The light brown, alcoholic solution was decolorized by boiling for five minutes with two successive portions of activated charcoal, filtering after each boiling. The combined charcoal residues were exhausted by boiling with three successive portions of alcohol, adding the combined alcoholic washings to the original solution. The solvent was removed by evaporation on a water bath.

The grayish, scaly residue obtained gave about 1 Gm. of a grayish white powder which started to decompose at 200°.

Twenty milligrams of this residue, suspended in normal saline solution and injected intraperitoneally into a 250-Gm. rat, killed the animal in one hour and thirty minutes with the same symptomatology previously described.

The residue was freely soluble in 95% alcohol and a 5% solution of potassium hydroxide. It was very slightly soluble in water, and the solubility was greatly decreased on acidification. In relatively high concentration it formed a viscous hydrophilic sol which showed gelation on cooling or standing. Even in very large dilution its aqueous solution frothed tremendously when shaken with air, or formed pasty long-lasting emulsions when shaken with oils or organic immiscible solvents.

The residue gave a positive test with the Molisch reagent and reduced Fehling's solution only after prolonged boiling with dilute hydrochloric acid.

It gave color reactions with the Liebermann-Burchard and Hager-Salkowski reagents for sterols.

It gave a display of color when dissolved in concentrated sulfuric acid changing from crimson to purple and finally to violet.

The residue had a range of decomposition temperature from 180° to 200°. It contained about 0.6% of nitrogen as determined by the micro Dumas method. The contaminant containing the nitrogen was separated chromatographically.

The deposit (*A*) obtained from the original alcoholic extract as well as the precipitate (*C*) obtained from the residue left by the evaporation of the aqueous solutions were separately examined. They both gave the same tests characteristic of saponin. It was concluded that they were identical and might be a single saponin or a mixture of water-soluble saponins of no significant toxicity.

The chloroform and ether washings, *W*1, and the ethereal extracts, *W*2, obtained from the large-scale extraction of the drug were combined, concentrated by distillation to about 300 cc., dehydrated with anhydrous sodium sulfate, filtered, and evaporated spontaneously to dryness.

The brown residue proved to be very slightly active (100 mg. suspended in normal saline and injected intraperitoneally into a 250-Gm. rat killed the animal in several hours). The slight activity was very probably due to a contamination with the toxic acidic saponin which was partially dissolved in these washings, especially considering the fact that the picture of death was more or less similar to that caused by the toxic saponin.

The residue was further purified with charcoal and repeated precipitation from other solutions by the addition of petroleum ether and from carbon tetrachloride solution by the addition of normal hexane. The acidic white amorphous, asbestos-like substance isolated melted at 159 to 162° and its molecular formula calculated as $C_4H_{11}O_4$. It was physiologically innocuous.

### Purification of the Toxic Principle

Using a 20% solution of ethyl alcohol in acetone as a developer, and pure acetone followed by a 25%, then 5% solution of alcohol in acetone as washer, the toxic residue was absorbed on a freshly ignited magnesium oxide column.

It appeared under the ultraviolet light as a subfringeal band with a greenish blue fluorescence.

Another hazy dispersed layer moving along with the washer solutions exhibited a slight greenish fluorescence.

While the distinct band moved very slowly on passing these washing solutions, the other fluorescent-dispersed substance moved rapidly and could be detected early in the first portions of the filtrate by its distinct bluish green fluorescence.

The elution of the distinct zone with boiling alcohol and the evaporation of the alcohol to dryness on a water bath, left the substance as a whitish, glistening, nitrogen-free residue.

The combined acetone washings were evaporated spontaneously and the oily nitrogenous residue exhibited a greenish fluorescence under the ultraviolet light.

When kaolin was used instead of magnesium oxide, the impurities were held behind while the substance passed along on washing with alcohol.

The substance was further purified by crystallization from 60% methyl alcohol. The long prismatic crystals with pyramidal ends which formed slowly, melted with decomposition at 212°. On recrystallization from methyl alcohol the decomposition temperature remained constant.

### Large-Scale Extraction

Ten kilograms of the dried powdered root was extracted according to the previous procedure in a Lloyd extractor and about 3 Gm. of the crystalline compound, m. p. 212°, was prepared.

The compound was freely soluble in 95% ethyl alcohol and methanol, soluble in acetone, slightly soluble in water, and very slightly soluble in dilute acids, ether, chloroform, carbon tetrachloride, petroleum ether, and benzene.

The compound exhibited in alcoholic solution a strong greenish blue fluorescence and has a characteristic absorption spectrum in the ultraviolet range. It had an $[\alpha]_D^{25} = +46°$ (ethyl alcohol). Its molecular formula was calculated as $C_{15}H_{20}O_{22}\cdot 2H_2O$.

The compound had no odor when inhaled but was extremely sternutatory. It has a delayed but marked acrid taste.

The compound was tested for the presence of phenolic, alcoholic, and carbonyl groups with negative results. It showed evidence of unsaturation.

It behaved as a weak acid and gave all the reactions characteristic of saponins.

It gave typical color reactions with the Liebermann-Burchard (12) and Hager-Salkowski (13) reagents for sterols.

It was thus identified as a steroid acidic saponin.

### Tests for Identity of the Compound, m. p. 212° (dec.)

1. When a few milligrams of the substance was dissolved in 2 cc. of glacial acetic acid containing a trace of ferric ion, and this solution was superimposed on 2 cc. of concentrated sulfuric acid containing a trace of ferric ion, two rings appeared at the junction of the two solutions; the upper one in the acetic acid layer was dark crimson in color, and the lower one in the sulfuric acid layer was rose-pink.

2. When a few milligrams of the substance was dissolved in concentrated sulfuric acid, a reddish color was developed which changed to crimson, purple, and finally to violet. The violet color was permanent for at least twenty-four hours. It disappeared on the addition of water.

## TOXICOLOGY

### Tests for Toxicity

Five milligrams of the compound suspended in 1 cc. of normal saline solution and injected intraperitoneally into a 30-Gm. mouse killed the animal in forty-five minutes with the following symptoms. Immediately after the injection, the animal showed extensive uresis and catharsis. During the following ten minutes it was extremely sensitive, showed sudden runs and jumps, and gradually fell into increasing drowsiness, becoming very sluggish, showing at the same time an increasing dyspnea. After thirty minutes following the injection, the dyspnea and slow heart were very pronounced and the animal started to lose sensation in its tail. This paralytic condition ascended, resulting in complete loss of function of the lower limbs.

Before the death of the animal, cutaneous sensibility was abolished, the heart slowed down, and the labored respiration became occasional and finally failed, resulting in the death of the animal.

Using a normal saline solution of the compound of *p*H 7.4 (which was preferred to the suspension in the parenteral administration) the above test was repeated with the same results.

In relatively larger doses the compound was found to be a potent convulsant.

### Determination of the Approximate *MLD*

The *MLD* of the compound was determined and found to be 0.13 mg. per Gm. body weight of mouse per hour.

### Determination of $MLD_{50}$

The $MLD_{50}$ of the compound was determined (Table I) and computed as 0.065 mg. per Gm. body weight of mouse per hour.

TABLE I.—SUMMARY OF THE RESULTS OBTAINED FOR THE DETERMINATION OF $MLD_{50}$

| Group No. | Total Number of Animals | Dosage, Mg./Gm. Body Wt. | No. of Dead Animals | No. of Surviving Animals | % Mortality |
|---|---|---|---|---|---|
| 1 | 10 | 0.150 | 10 | 0 | 100 |
| 2 | 10 | 0.125 | 10 | 0 | 100 |
| 3 | 10 | 0.100 | 8 | 2 | 80 |
| 4 | 10 | 0.075 | 7 | 3 | 70 |
| 5 | 10 | 0.050 | 2 | 8 | 20 |
| 6 | 10 | 0.025 | 0 | 10 | 0 |

The symptomatology showed that the compound in sublethal doses had a stimulant action at first, followed by a depression (14).

With lethal doses the compound showed marked depressant action, especially on circulation and respiration (10, 14, 15) and, prior to the death of the animal, peculiar ascending paralytic symptoms (6).

In relatively larger doses the compound was found to be a potent convulsant (16).

### Determination of the Hemolytic Activity

The compound was found to have a strong hemolytic action.

The hemolytic index (17) of the compound as determined on dog's erythrocytes (washed) was found to be 1:15,000.

### The Compound as a General Protoplasmic Poison

**1. Effect on Plant Tissues.**—A study similar to the phytopharmacological study of Macht (10) demonstrating the retarding poisonous effect of the fluidextract of poke root on the seedlings of *Lupinus albus*, was carried out using aqueous solutions of the pure compound and the seedlings of *Pisum sativum*.

The results obtained showed that the compound exerted a stimulant effect on the growth of the roots of *Pisum sativum* in concentrations below 12.5 p. p. m. (Table II).

Above this concentration the compound showed a definite and increasing retarding effect on the growth of the roots until a concentration of 100 p. p. m. was reached when the growth of the root was completely stopped.

When the dose was increased to 10 mg. (by the intravenous injection of another cc. of 0.5% solution), a marked depression of both blood pressure and respiration was produced which lasted for ten minutes. Slow recovery followed.

When the dose was increased to 15 mg. (by a third intravenous injection of 1 cc. of 0.5% solution), a serious fall in blood pressure and respiration took place in less than two minutes resulting in the

TABLE II.—DETERMINATION OF THE PHYTOTOXIC INDEX OF THE COMPOUND. MELTING POINT 212°

| Tumbler No. | Ml. of 0.5% Solution of the Compound per Tumbler | Effective Concn. of the Compd. in P. P. M. | Av. Original Length of Roots in Mm. | Av. Length of Roots After 24 Hours | Increment Increase in Growth in 24 Hours | Phytotoxic Index[a] |
|---|---|---|---|---|---|---|
| Control | 0 | 0 | 48.1 | 65.8 | 15.7 | 100 |
| I | 0.25 | 6.242 | 47.4 | 64.3 | 16.9 | 107.3 |
| II | 0.5 | 12.46 | 50.8 | 72.5 | 21.7 | 138.2 |
| III | 1 | 24.87 | 44.7 | 51.8 | 7.1 | 38.8 |
| IV | 2 | 49.50 | 47.1 | 51.1 | 4.1 | 26.1 |
| V | 3 | 73.89 | 44.7 | 47.8 | 3.1 | 19.7 |
| VI | 4 | 98.04 | 51.5 | 51.1 | −0.4[b] | −2.58 |
| VII | 5 | 121.99 | 53.8 | 53.4 | −0.4 | −2.58 |

[a] Growth increment for twenty-four hours expressed as percentage of corresponding increment in control test. The lower the phytotoxic index, the greater the toxic effect.
[b] Negative results probably due to shrinkage as a result of dehydration.

**2. Effect on Protozoa.**—The compound caused an immediate loss of motility and subsequent death of *Paramecium caudatum* (*Infusoria-Ciliata*) in a dilution of 1:7500.

**3. Effect on Gold Fish.**—Gold fish placed in 0.001% aqueous solution of the compound turned over on their sides in about twenty minutes. Death occurred a few minutes later.

These results evidently indicated the protoplasmic poisonous effect of the compound.

### The Compound as an Irritant

**1. To the Respiratory Tract.**—The compound on inhalation caused extreme sternutatory effect with immediate and lasting nasal discharge. After this effect subsided the following symptoms were manifested for several hours: rhinitis, pharyngitis, sore throat, cough with pains in the chest, and persistent headache.

In severe cases, all the symptoms of a severe cold were produced, lasting for at least two days with pains in the chest and eyeballs, headache accompanied by nausea and loss of appetite (18, 19).

**2. To the Gastrointestinal Tract.**—The compound was found to produce marked catharsis in rats only when administered in relatively large doses (10, 15, 18, 20).

It was also noticed that the compound was slightly absorbed when administered orally.

### Action of the Compound on Circulation and Respiration (10, 15)

One cubic centimeter of 0.5% normal saline solution of the compound (*p*H 7.4) when injected intravenously into a 4-Kg. rabbit with intact vagi under ether anesthesia produced a slight excitation of the respiration and slight lowering of the blood pressure. Both functions were restored to normal after a few minutes.

paralysis of both functions and the subsequent death of the animal.

This preliminary experiment showed the marked depressant action of the compound on both circulation and respiration. This effect needs a detailed quantitative pharmacological study.

### Isolation of a Water-Soluble Saponin

A detailed procedure for the isolation of a water-soluble saponin was given.

Since the finished product was of a doubtful purity, no microanalysis or constants were determined.

On hydrolytic cleavage with dilute hydrochloric acid it gave a steroid sapogenin very probably isomeric, if not identical, with sarsapogenin of the general formula $C_{27}H_{40}O_3$.

The sapogenin melted at 179–181°.

The sugar moiety was identified as rhamnose.

## SUMMARY

1. The extraction of the dried powdered root of *Phytolacca americana* L. according to the successive solvent extraction method, the general lead method, and the Stas-Otto method did not establish the presence of any alkaloid but established the presence of the following substances: starch, gum, oxalates, hemicellulose, steroid materials, and at least one water-soluble saponin.

2. Pharmacological tracing of the physiologically active constituent of the root was carried out.

This tracing ultimately led to a highly toxic acidic steroid saponin of the molecular formula

$C_{38}H_{66}O_{22} \cdot 2H_2O$, whose physiological activity was correlated, in all respects, to the reported physiological activity of the root and its crude extracts.

3. Methods of isolation, purification, and identification of the toxic compound are given.

4. The compound of the molecular formula $C_8H_{12}O_4$, m. p. 159–162°, was isolated from the ether-chloroform solutions of the water-insoluble portion of the alcoholic extract of the drug. It was found to be pharmacologically inactive.

5. The sapogenin of the water-soluble saponin, m. p. 179–181°, was found to be of the general formula $C_{27}H_{44}O_7$, and the sugar moiety was identified as rhamnose.

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# The Muscle Relaxant Effects Produced By *Potentilla anserina* Extracts. I. Fractionation Studies*,†

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**Preliminary studies of the muscle relaxant effects produced *in vitro* by ethyl and methyl acetate fractionation of *Potentilla anserina* extracts are reported. This activity has been compared with that of papaverine hydrochloride. There is evidence that the activity is produced by a glycoside or mixture of glycosides present in largest amounts in acetate extractives of the drug.**

THE LITERATURE during various periods of the past fifteen years has reported on the use of drug preparations of the above-ground portions of *Potentilla anserina* L. in the treatment of essential dysmenorrhea. Haupstein (1) and Bliss (2) employed dried water extracts of the drug in clinical cases of dysmenorrhea and both investigators reported that a complete relief of pain was obtained by some patients and a partial relief by others following oral administration. Schneider and Nevinny (3), Haupstein (1), and Youngken (4) found uterine stimulant activity was produced *in vitro* with water, petroleum ether, and alcoholic extractives of the drug. Although the first two workers had also reported that a muscle relaxant effect was produced against an induced barium spasm using concentrated water decoctions, Youngken (4) could not confirm this double activity. It was therefore deemed necessary to reinvestigate the drug for its effect on muscle tissues and to isolate, if possible, the principles responsible for activity. In this paper a relaxant effect heretofore not confirmed is described, and the nature of the extract fractions wherein it occurs in significant quantities is reported.

## EXPERIMENTAL

More than 60 lb. of the above-ground portions of *Pontentilla anserina* L. were obtained from a commercial source and 10 lb. collected from the Red River Valley section of Minnesota. They were authenticated and powdered (No. 40 mesh) for extraction.

Intestinal and uterine longitudinal strips from rats and guinea pigs weighing from 85 Gm. to 100

* Received April 30, 1949, from the School of Pharmacy and Dept. of Chemistry, University of Washington, Seattle, Wash.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.
† This study has been supported through a fellowship established by the International Cellucotton Products Corporation, Chicago, Ill., in the Department of Pharmacognosy, University of Washington, Seattle 5, Wash.

Gm. and 225 Gm. to 385 Gm., respectively, were selected for the preliminary tests. These were removed immediately from the sacrificed animal and placed into a muscle chamber with Locke-Ringer's solution. The chamber and solution, properly oxygenated, were kept at a constant temperature of 38°.

Solvent extracts of powdered *Potentilla anserina* were prepared with the use of acetone, ether, water, alcohol, moist ethyl and methyl acetates. These were employed with continuous extraction methods and by direct agitation. In each case attempts were made to effect crystallization or purification of the constituents by fractionation methods.

**Acetone Fractionation.**—Seventy-one grams of powdered drug was extracted in a continuous Soxhlet apparatus for eighteen hours; the extract concentrated to dryness, then taken up with acetone and filtered. The filtrate was freed of tannins with lead subacetate, further concentrated to dryness, washed with water, taken up with 60% alcohol, and crystallized for tormentol (5). A total of 100 mg. of tormentol was obtained. These crystals melted between 200–208°. When tested on muscle strips *in vitro* they produce no significant activity. Filtrate and residue fractions of the acetone extraction likewise failed to produce muscle response. It was therefore concluded that acetone extractions of the drug possess no significant action *in vitro*.

**Ninety-five % Ethanol Fractionation.**—One hundred and twenty grams of powdered drug was extracted in a continuous Soxhlet extractor with 95% ethyl alcohol for seventy hours. An insoluble brown resinous residue was separated and the filtrate concentrated to a deep red viscous syrup with a sugar odor. When tested on isolated muscle strips this water-soluble syrupy extract fraction produced a response similar to that previously reported (i.e., initial relaxation followed by stimulation)(3). In comparison, however, the initial relaxant effect *in vitro* was quite pronounced. This was repeated several times and considered significant. Further fractionation was therefore attempted. The relaxant effect lasted for from one to two minutes. This was then almost immediately overcome by a recovery of rhythmic contractions followed by marked stimulation of muscle tone. The tannin-free alcoholic fraction produced the same general effects. There was evidence of crystal formation upon subsequent fractionation using acetates. This will be referred to subsequently under "acetate fractionation."

**Water-Extractive Fractionation.**—Approximately 158 Gm. of powdered drug was extracted with water for a period of from eighteen to twenty hours with continuous agitation. This extractive was then further partitioned with water, alcohol, acetone, and ether until 4.4 Gm. of a water-soluble residue was obtained. When tested this extract produced very much the same response as observed following the use of the alcoholic extraction above. Attempts to crystallize the components of the extract yielded crystalline material of a hygroscopic nature which was mixed with a reddish amber-colored syrup. There was further evidence that the presence of sugars contributed in large measure to overcoming the observed relaxant effects. The fraction was set aside for further examination.

**Acetate Fractionation.**—On the basis of the consistent depressing effect of short duration as observed in alcohol and water extracts and the fact that the literature has already reported evidence for glycosides (6, 7) in the crude drug, it was thought advisable to employ acetate solvents for further fractionation studies. Ethyl and methyl acetate solvents are frequently employed for glycoside separations. Powdered drug material (No. 40 mesh) was then extracted with moist ethyl acetate at room temperature in a Soxhlet extractor for from fifteen to eighteen hours. The solvent was removed from the extract under diminished pressure and residue extracted repeatedly with hot water. Tannins were removed from the solution by means of lead subacetate; the excess lead separated by $H_2S$ and the residue further concentrated under reduced pressure. From 800 Gm. of powdered drug, 12 Gm. of a clear reddish brown, viscid, water-soluble extract was obtained. Pronounced muscle relaxation responses were observed following injections of quantities of this fraction into the muscle chamber. It was therefore tested further. In dilutions of 1:800 to 1:1000 the relaxant response lasted from thirty to sixty minutes or longer. Tissues regained their normal activity in rate and amplitude upon washing out. This activity was then compared with that produced by papaverine hydrochloride. Papaverine was selected on the basis of its use as a standard muscle spasmolytic after the methods of Munch (8) and Grote (9). It was observed that the ethylacetate fraction was comparable to the activity of between 100 and 300 $\gamma$ of papaverine or approximately 2.85 $\gamma$ papaverine per cc. of Ringer Solution.

Such a response led to further fractionation studies on the ethyl acetate residue. This residue was then dissolved in moist ethyl acetate and extracted repeatedly by water in a separatory funnel. The acetate layer was separated and concentrated to dryness and tested. It had no activity. The aqueous layer was then concentrated under reduced pressure to yield a water-soluble white crystalline-like substance. This aqueous layer produced significant muscle relaxation responses when tested *in vitro* (Fig. 1). The average yield of it was 4% to 5%. The crystalline substance was difficult to effect in a pure crystalline state due to its hygroscopic nature.

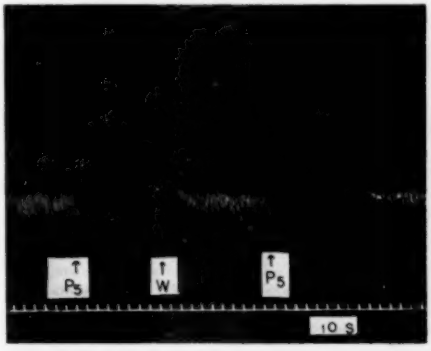


Fig. 1.—Rat intestine (*in vitro*).
$P_5$, *Potentilla* glycoside fraction, 1:3500; *W*, Wash. Time, 10 seconds.

In fact it decomposed somewhat upon prolonged heating. Nevertheless tests were made on its myotropic effects and its chemical properties. From preliminary testing, this fraction has been found to be positive to Fehling's solution after boiling for one minute. It was positive to Molisch test (deep violet) and positive to Tollens' test on heating (black precipitate). It formed an osazone after boiling for one and one-half hours; the osazone was identified as glucosazone using the phenolhydrazine test (10). On the basis of these preliminary tests it is felt that there are indications for the presence of a glycoside or mixture of glycosides in the fraction. The fraction was therefore designated a "glycoside" fraction. Tests made for the presence of cyanogenic and phenol glycosides with the barium hydroxide fusion test (11) and ferric chloride test have thus far been negative.

Muscle relaxant effects produced by this glycoside fraction were measured against induced barium spasms *in vitro* in which intestinal and uterine segments from rats and guinea pigs were employed. When a spasm was induced by 0.5 cc. of a 1% $BaCl_2$ solution (1:70,000 in the chamber) it would be effectively released by dilutions of from 1:3500 to 1:4500 of the dried glycoside portion of the moist ethyl acetate fraction (Figs. 2 and 3). Often the barium spasm failed to take effect if a muscle had been previously prepared with the *Potentilla* glycoside extract. As well as could be determined quantitatively, within tolerants of biological testing, glycosidic extracts were comparable in spasmolytic activity to approximately that of 300–600 γ papaverine HCl.

Observations on a large number of uterine and intestinal strips *in vitro* showed consistent antispasmodic effects with the use of moist methyl acetate extracts prepared and employed in the same manner as of the moist ethyl acetate fractions. When compared quantitatively, however, it was found that moist methyl acetate fractions were considerably less in their potency. Likewise total extract yields were approximately one-third the amount of the ethyl acetate extraction. Otherwise, chemical tests indicated the presence of the same glycosidic components.

Attempts were then made to observe the effect of hydrolysis upon the activity of the glycoside fraction. It was thus hydrolyzed with 2% HCl for twelve hours on a steam bath and then extracted with ether. Ether layers were devoid of the relaxant activity when tested on muscle strips. This fraction likewise gave negative reactions to Fehling, Molisch, $FeCl_3$, and Liebermann tests. Aqueous fractions from the partitioned ether extract were then tested physiologically on muscle strips and by the same chemical tests. These fractions were found to possess slight relaxation effects. When tested for evidence of glycoside, results were positive. Glucosazine was obtained in crystalline form. It was therefore concluded following repeated extraction and hydrolysis that the activity thus described is diminished somewhat, but not entirely, by hydrolysis and that it resides in aqueous fractions. Further, that it is present in largest quantities in aqueous partitions of moist ethyl acetate extracts. Subsequent partitioning of this fraction during which alcohol solvents were employed indicated that the glycoside activity was lost in concentrations of 80% alcohol or more.

From repeated tests for sugars there are indications that the presence of glucose residues lends evidence for the presence of this sugar in large quantities in hydrolysates of acetate fractions.

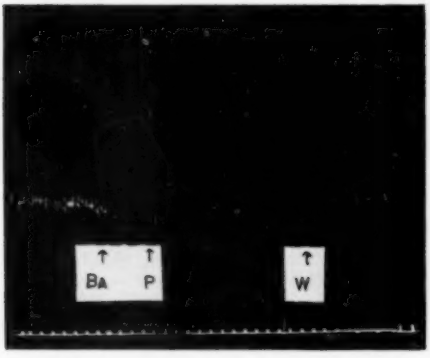


Fig. 2.—Rat intestine (*in vitro*).
Ba, $BaCl_2$ 1:70,000; *P*, *Potentilla* glycoside fraction, 1:3500; *W*, Wash. Time, 10 seconds.

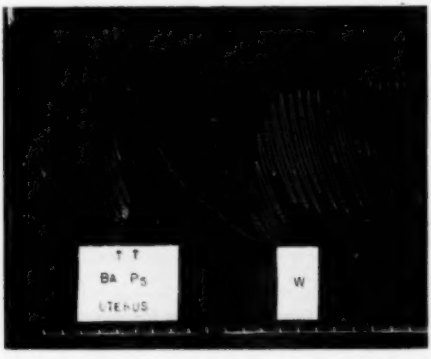


Fig. 3.—Virgin rat uterus (*in vitro*).
Ba, $BaCl_2$ 1:35,000; $P_5$, *Potentilla* glycoside fraction 1:3500; *W*, Wash. Time, 1 minute.

## SUMMARY

Studies of the intestinal and uterine musculature response *in vitro* of certain *Potentilla anserina* extracts have confirmed that there is a spasmolytic principle present in the drug.

Significant muscle relaxant effects have been produced experimentally following the use of moist ethyl and methyl acetate fractions. This activity has been compared with that of papaverine hydrochloride. Evidence is established for

the presence of a glycoside or a mixture of glycosides in these fractions.

Preliminary tests using isolated muscle strips indicate that this glycoside mixture is responsible for the observed relaxant effects. There is evidence that, as the glycoside fraction is hydrolyzed, the depressant activity is diminished.

Alcoholic (above 80 per cent), ether, and acetone extracts do not contain the depressant principle in significant amounts. It is thought that the presence of sugars in water extracts tends to conceal the glycoside activity.

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# The Metabolism and Toxicity of Salicylic Acid in Combination with Various Drugs*

By GEORG CRONHEIM, JULIUS WILAND, and MICHAEL EHRLICH

**Using rabbits and dogs as test animals, combinations of sodium salicylate with various antacids have been investigated for toxicity and salicylate absorption and excretion. The results of a series of experiments are reported.**

IN RECENT YEARS following the study by Coburn (1) regarding the value of massive doses of sodium salicylate in the treatment of rheumatic fever, interest in the pharmacology and toxicology of salicylates has reawakened and has led to a number of important observations. These investigations were facilitated by the work of Brodie, *et al.* (2), and Smith, *et al.* (3), which made available simple methods for the determination of salicylic acid and some of its metabolic derivatives in various body fluids.

One of the difficulties in the use of salicylates, particularly in large doses, is the appearance of gastric disturbances often followed by other and more severe symptoms. It seems well established that the former is caused partly by a direct action of free salicylic acid on the gastric mucosa and partly by an emetic reflex through the central nervous system (Goodman and Gilman) (4). In order to reduce these untoward effects, sodium bicarbonate is frequently administered simultaneously. However, it has been found (Smull, *et al.*) (5) that this alkalizer lowers the blood salicylate level quite considerably and thus counteracts the efficacy of the salicylate therapy.

In the present study, another approach has been investigated, namely, the combination of sodium salicylate with adsorptive gastric antacids such as aluminum hydroxide and magnesium trisilicate. While these antacids will definitely raise the *p*H of the gastric fluid, their effect on the absorption of salicylates from the gastrointestinal tract and the blood salicylate levels under these conditions had not been reported.[1]

A few experiments were also made with addition of "detoxifying" agents in the form of sodium and calcium ascorbate. It had been reported by Thompson and Dragstedt (7) and by Schnedorf, Bradley, and Ivy (8) that calcium ions in the form of calcium gluconate will raise in dogs the tolerance threshold for toxic symptoms following oral doses of salicylates. Since it is also known that the human requirement for ascorbic acid is noticeably increased when salicylates are administered (9–11), and since ascorbic acid is credited with certain "detoxifying" properties, it seemed of interest to combine all these observations and study the effect of sodium and calcium ascorbate upon the metabolism and toxicity of sodium salicylate.

* Received March 1, 1949, from The Research Department of The S. E. Massengill Company, Bristol, Tenn.

[1] While this investigation was in progress, Hoffman, *et al.* (6), reported their observations on the clinical use of aspirin combined with aluminum hydroxide.

## METHODS

The experiments were carried out in rabbits and dogs. The drugs were administered in water (10 and 15 ml., respectively) by stomach tube followed by 5 and 10 ml., respectively, of plain water.

The salicylate blood levels were determined according to the method of Brodie (*loc.cit.*), but using smaller volumes requiring only 0.1 ml. of whole blood for each analysis. The color of the final solution was read in a Coleman Universal Spectrophotometer set at 540 mμ and using filter PC-4.

The determinations of free and conjugated salicylic acid in urine were made according to the procedure of Smith (*loc.cit.*). We have rechecked the experiments leading to the formulas for the calculation of salicylic, salicyluric,[1] and salicylglucuronic acids and found essentially the same conversion factors. However, it should be mentioned that the values given in Smith's paper as well as in the following tables indicate the amounts of salicylic acid as present in the conjugated form and not the amount of the conjugated salicylic acid derivatives. The urine specimens have to be diluted so that they contain not more than 0.5 mg. of salicylic acid in the sample. The only discrepancy between Smith's and our results was in the rate of hydrolysis of salicyluric acid. According to Smith, the hydrolysis after three and six hours reaches 34% and 55%, respectively. We found repeatedly values of 79% and 95%, respectively.

It was noted that the colorimeter readings always indicated an apparent loss of salicylic acid in the test for total salicylates. A detailed check of the procedure showed that a very small amount of the concentrated hydrochloric acid is carried through the ethylene dichloride into the final solution. Since the color of ferric salicylate solution is greatly affected by even slight changes in *p*H, the apparent loss could be explained by variations in the acidity and the necessary corrections could be applied.

**Acute Toxicity.**—In order to determine acute toxicity, single doses of 1 Gm./Kg. of sodium salicylate were administered alone or in combination with other drugs to fasting rabbits and the number of surviving animals was recorded. The observation period was set at twenty-four hours, since no acute effects of the drug are found after this time. The results (Table I), while not very definite on the basis of a statistical analysis, seem to indicate that both sodium bicarbonate and ascorbic acid reduce to some extent the acute toxicity of salicylic acid while magnesium trisilicate is without any effect.

TABLE I.—ACUTE TOXICITY IN RABBITS

| Drugs Administered | Mortality |
|---|---|
| Na Salicylate 1 Gm./Kg. | 4/12 = 33% |
| Na Salicylate 1 Gm./Kg. + Na Bicarbonate 0.19 Gm./Kg. | 1/12 = 8% |
| Na Salicylate 1 Gm./Kg. + Mg Trisilicate 0.4 Gm./Kg. | 5/12 = 42% |
| Na Salicylate 1 Gm./Kg. + Mg Trisilicate 0.4 Gm./Kg. + Na Ascorbate 0.5 Gm./Kg. | 2/12 = 17% |
| Na Salicylate 1 Gm./Kg. + Mg Trisilicate 0.4 Gm./Kg. + Ca Ascorbate 0.5 Gm./Kg. | 0/12 = 0 |

An attempt was made to determine the acute toxicity in fasting dogs, using the occurrence of vomiting as an indicator. However, the results were so irregular that no conclusions could be drawn.

**Blood Levels after Single Doses.**—The absorption of salicylic acid was studied in fasting rabbits by hourly blood determinations after single doses of 1.0 and 0.5 Gm./Kg., respectively, of sodium salicylate without and with various antacids. The results (Table II) are in one respect rather unexpected: the blood levels for the first two hours are practically the same for both the larger and the smaller dose of sodium salicylate.

A study of the individual figures reveals some interesting details. After the larger dose, the variations between different animals are greater than after the smaller dose. Furthermore, while the time curve after the smaller dose shows in 5 out of 6 animals, the expected drop in salicylate blood level after four hours, this decrease is found only in 1 out of 6 animals after the larger dose. It is obvious that considerably more data are required to be sure that these differences are statistically significant. If so, it would indicate that after the larger dose the absorption of salicylic acid from the gastrointestinal tract is markedly disturbed, possibly due to local irritation. Such an assumption is supported by the observation that all animals that died in the acute toxicity studies showed gross pathological changes of the stomach mucosa.

The influence, if any, of the various antacids on the salicylate blood level is difficult to ascertain especially after the larger doses. With the smaller doses, the data are much more uniform and consistent, and the conclusion seems justified that all antacids used reduce the concentration of salicylic acid in blood.

A few experiments (Table III) were also made on dogs after a single oral dose of 300 mg./Kg. of sodium salicylate alone or in combination with 120 mg./Kg. of magnesium trisilicate. The results showed in every instance a lower blood salicylate concentration when this antacid was administered.

**Blood Levels after Repeated Doses.**—Since the main interest in the therapeutic use of salicylates lies in their prolonged administration, the absorption and excretion of salicylic acid was studied after repeated doses of the drug either alone or in combination.

In the rabbit experiments (4 animals in each group), the drugs were given to nonfasting animals every morning by stomach tube. Blood samples were taken after three and twenty-four hours and urine collected every twenty-four hours. The dose of the drug was 0.5 Gm./Kg. of sodium salicylate alone or with 0.2 Gm./Kg. of magnesium trisilicate.

In the dog experiments each nonfasting animal received the drug twice daily at 8:30 a. m. and 2:30 p. m. Blood samples were taken at 8:30 a. m., 9:30 a. m., and 3:30 p. m. Urine was collected at 8:00 a. m. Each dose consisted of 0.15 Gm./Kg. of sodium salicylate with either 0.05 Gm./Kg. of sodium bicarbonate or 0.06 Gm./Kg. of magnesium trisilicate. Each experiment lasted five days in two successive weeks. Two dogs had sodium bicarbo-

[1] We are indebted to Dr. Brodie and Dr. Udenfriend, New York University, for a sample of salicyluric acid. It gave the same results as one prepared in our laboratories by P. Lucas.

TABLE II.—SALICYLATE BLOOD LEVELS IN RABBITS AFTER SINGLE DOSE

| Drug given | | Salicylate Blood Level, Mg. % | | | | | | Average |
|---|---|---|---|---|---|---|---|---|
| Sodium Salicylate 1 Gm./Kg. | 1 hr. | 53 | 43 | 50 | 47 | 63 | 53 | 51.5 |
| | 2 hr. | 65 | 50 | 52 | 52 | 64 | 50 | 55.5 |
| | 4 hr. | 67 | 47 | 56 | 54 | 64 | 50 | 56.3 |
| | 6 hr. | 66 | 46 | 50 | 51 | D[a] | D[a] | 53.3 |
| Sodium Salicylate 1 Gm./Kg. Sodium Bicarbonate 0.19 Gm./Kg. | 1 hr. | 46 | 58 | 44 | 62 | .. | .. | 52.5 |
| | 2 hr. | 50 | 52 | 39 | 64 | .. | .. | 50.1 |
| | 4 hr. | 46 | 57 | 36 | 53 | .. | .. | 46.5 |
| | 6 hr. | 45 | 47 | 33 | 48 | .. | .. | 43.3 |
| Sodium Salicylate 1 Gm./Kg. Magnesium Trisilicate 0.4 Gm./Kg. | 1 hr. | 60 | 61 | 65 | 66 | .. | .. | 63.0 |
| | 2 hr. | 59 | 60 | 71 | 69 | .. | .. | 64.8 |
| | 4 hr. | D[a] | 53 | D[a] | D[a] | .. | .. | .. |
| | 6 hr. | .. | 45 | .. | .. | .. | .. | .. |
| Sodium Salicylate 0.5 Gm./Kg. | 1 hr. | 60 | 62 | 61 | 62 | 50 | 50 | 57.5 |
| | 2 hr. | 56 | 68 | 54 | 58 | 50 | 49 | 55.8 |
| | 4 hr. | 47 | 64 | 50 | 50 | 44 | 47 | 50.0 |
| | 6 hr. | 37 | 56 | 46 | 44 | 41 | 40 | 44.0 |
| Sodium Salicylate 0.5 Gm./Kg. Sodium Bicarbonate 0.1 Gm./Kg. | 1 hr. | 62 | 41 | 50 | 40 | 47 | .. | 48.0 |
| | 2 hr. | 59 | 41 | 50 | 38 | 57 | .. | 48.0 |
| | 4 hr. | 50 | 40 | 33 | 37 | 46 | .. | 40.1 |
| | 6 hr. | 50 | 38 | 30 | 35 | 44 | .. | 39.4 |
| Sodium Salicylate 0.5 Gm./Kg. Magnesium Trisilicate 0.2 Gm./Kg. | 1 hr. | 35 | 48 | 50 | 38 | 50 | 42 | 43.8 |
| | 2 hr. | 38 | 38 | 52 | 36 | 47 | 41 | 42.0 |
| | 4 hr. | 30 | 29 | 46 | 32 | 44 | 39 | 36.7 |
| | 6 hr. | 30 | 20 | 41 | 26 | 38 | 35 | 31.7 |
| Sodium Salicylate 0.5 Gm./Kg. Aluminum Hydroxide 0.1 Gm./Kg. | 1 hr. | 43 | 47 | 40 | 41 | 38 | .. | 41.8 |
| | 2 hr. | 44 | 48 | 40 | 44 | 35 | .. | 42.2 |
| | 4 hr. | 43 | 43 | 31 | 32 | 35 | .. | 36.8 |
| | 6 hr. | 32 | D[a] | 22 | 32 | 29 | .. | 28.8 |

[a] D = Animal died.

TABLE III.—AVERAGE SALICYLATE BLOOD LEVELS IN DOGS AFTER SINGLE DOSE

| | No. Animals | Salicylate Blood Levels in Mg. % after 1 Hr. | 2 Hr. | 4 Hr. | 6 Hr. |
|---|---|---|---|---|---|
| Sodium Salicylate 0.3 Gm./Kg. | 3 | 39 | 36 | 28 | 28 |
| Sodium Salicylate 0.3 Gm./Kg. + Magnesium Trisilicate 0.12 Gm./Kg. | 3 | 34 | 29 | 24 | 22 |

nate as the antacid during the first and magnesium trisilicate during the second week, while the other two dogs received the drugs in reverse order.

The rabbits receiving sodium salicylate plus magnesium trisilicate showed after the first dose a lower salicylate blood level than those receiving only sodium salicylate (Table IV). This is in agreement with the previously described single dose tests. The observation that the three-hour blood level on the first day is lower than that found in the single dose test (Table II) is in our opinion due to the fact that the animals in the single dose test had been fasted for eighteen hours prior to and also during the experiment. Starting with the second day, the addition of magnesium trisilicate resulted without exception in a higher salicylate level in the three-hour samples, while in the twenty-four-hour specimens no differences were found. Thus, in rabbits the addition of magnesium trisilicate has no detrimental effect upon the absorption of sodium salicylate from the gastrointestinal tract.

All animals in both groups showed the highest salicylate blood levels on the second and third days, followed by a decline on the fourth and fifth days. It will require further studies to determine whether this is an indication of a cumulative effect and a subsequent development of some equilibrium or tolerance.

In dogs the salicylate blood levels in the sodium bicarbonate group were always slightly lower than in the magnesium trisilicate group (Table V). In addition all animals showed a uniform drop of the salicylate blood level on the second day of the experiment. While the underlying mechanism is probably different from that in rabbits, it seems to indicate again some form of adjustment of the body to repeated doses of sodium salicylate.

**Urinary Excretion.**—The urinary excretion of free salicylic acid in rabbits is shown in Table VI. The recovery during the observation period amounts to 39% and 34% of the administered salicylic acid. However, since urine collection was stopped twenty-four hours after the last dose of the drug, it may be assumed that the total recovery of free salicylic acid would have reached from 40% to 45%.

Several attempts were made to determine conjugated salicylic acids in rabbit urine. Using ethylene dichloride and carbon tetrachloride as solvents for the differential estimation of salicyluric acid, the colorimeter readings indicated a complete absence of this compound. When a larger amount of urine from two rabbits that had received only sodium salicylate was treated according to the procedure of Kapp and Coburn (12) the ether-soluble

TABLE IV.—SALICYLATE BLOOD LEVELS IN RABBITS (4 ANIMALS IN EACH GROUP) AFTER REPEATED DOSES

| | 1st Day | 2nd Day | 3rd Day | 4th Day | 5th Day | Average |
|---|---|---|---|---|---|---|
| Daily Dose | Sodium Salicylate 0.5 Gm./Kg. | | | | | |
| | Salicylate Blood Level in Mg. % | | | | | |
| 3-Hr. Sample | 37 | 47 | 52 | 41 | 37 | 43 |
| 24-Hr. Sample | 9 | 17 | 14 | 8 | 9 | 12 |
| Daily Dose | Sodium Salicylate 0.5 Gm./Kg. plus Magnesium Trisilicate 0.2 Gm./Kg. | | | | | |
| | Salicylate Blood Level in Mg. % | | | | | |
| 3-Hr. Sample | 29 | 62 | 59 | 43 | 45 | 48 |
| 24-Hr. Sample | 7 | 19 | 13 | 9 | 9 | 12 |

TABLE V.—SALICYLATE BLOOD LEVELS IN DOGS (4 ANIMALS IN EACH GROUP) AFTER REPEATED DOSES

| | 1st Day | 2nd Day | 3rd Day | 4th Day | 5th Day | Average |
|---|---|---|---|---|---|---|
| Each dose containing | Sodium Salicylate 0.15 Gm./Kg. plus Sodium Bicarbonate 0.05 Gm./Kg. | | | | | |
| | Salicylate Blood Level in Mg. % | | | | | |
| One hour after first dose | 23 | 19 | 22 | 31 | 32 | 25 |
| One hour after second dose | 36 | 20 | 27 | 38 | 35 | 31 |
| 24 hours after first dose | 16 | 16 | 18 | 20 | 26 | 19 |
| Each dose containing | Sodium Salicylate 0.15 Gm./Kg. plus Magnesium Trisilicate 0.06 Gm./Kg. | | | | | |
| | Salicylate Blood Level in Mg. % | | | | | |
| One hour after first dose | 26 | 20 | 30 | 36 | 29 | 28 |
| One hour after second dose | 38 | 23 | 31 | 40 | 36 | 34 |
| 24 hours after first dose | 16 | 24 | 18 | 20 | 23 | 20 |

material proved to be exclusively salicylic acid. This was established by its melting point, by its mixed melting point with pure salicylic acid, and by a colorimetric comparison with salicylic acid. This leads to the conclusion that rabbits do not form salicyluric acid.

While it was possible to demonstrate the absence of salicyluric acid in rabbit urine, the question of other forms of conjugated salicylic acid has to be left open. The results of colorimetric tests after hydrolysis with concentrated hydrochloric acid were so unsatisfactory and scattered that no conclusions can be reached at the present time.

In the dog experiments, the urinary excretion of salicylic (SA) and salicyluric (SU) acids as well as total salicylates (ST) was determined according to Smith (*loc. cit.*) (Tables VII and VIII). It can be seen that the excretion was greatest on the second day of the experiment. This coincided with the low salicylate blood levels during this period. The total amount of sodium salicylate given to the four dogs in each experiment was 48.8 Gm. = 42.1 Gm. of salicylic acid. Thus the total recovery of salicylic acid in urine accounts for 48% in the sodium bicarbonate group and 59% in the magnesium trisilicate group. These figures are again too low since the urine collection was stopped eighteen hours after the last dose of the drug. The total recovery of salicylates in these experiments can be assumed to reach at least 55% and 65%, respectively.

TABLE VI[a]

| Drug Administered | Sodium Salicylate | Sodium Salicylate plus Magnesium Trisilicate |
|---|---|---|
| Total weight of four rabbits | 13.24 Kg. | 14.24 Kg. |
| Total amount of salicylic acid given | 28.6 Gm. | 30.5 Gm. |
| Total daily excretion of free salicylic acid | | |
| 1st day | 2.82 Gm. | 1.83 Gm. |
| 2nd day | 1.24 Gm. | 2.18 Gm. |
| 3rd day | 2.43 Gm. | 2.79 Gm. |
| 4th day | 2.61 Gm. | 2.10 Gm. |
| 5th day | 2.09 Gm. | 1.54 Gm. |
| Total excretion | 11.19 Gm. | 10.44 Gm. |
| Recovery | 39% | 34% |

[a] Urinary excretion of free salicylic acid by four rabbits in each group, receiving daily 0.5 Gm./Kg. of sodium salicylate alone or with 0.2 Gm./Kg. of magnesium trisilicate.

The distribution of the various salicylate fractions indicates a fairly large amount (38%) of free salicylic acid in both groups, while the amount of salicyluric acid accounts for only 15% and 10%, respectively. It seems doubtful that the difference in these last two figures is significant, since the daily variations are fairly wide. It should also be noted that the magnesium trisilicate group shows a reversal of the SA and SU figures on the first day. While this is not an explanation, it is certainly a further indication of the previously mentioned belief that some form of adjustment takes place in the dogs to the repeated administration of sodium salicylate.

It may be mentioned that the average urinary *p*H of 7.0 for the sodium bicarbonate group and 6.6 for the magnesium trisilicate group does apparently not reveal any difference in the metabolism of salicylic acid as indicated by the urinary excretion of the various salicylate fractions. This observation is different from that reported by Smith, *et al.* (*loc. cit.*, p. 249), but their experiments were made in humans and extended only over a relatively short period of time. In a few experiments where patients with acute arthritis or sciatica received sodium salicylate and magnesium trisilicate, it was found that the

TABLE VII[a]

| | 1st Day | 2nd Day | 3rd Day | 4th Day | 5th Day | Total |
|---|---|---|---|---|---|---|
| | | | Excretion in Gm. | | | |
| Free Salicylic Acid (SA) | 1.05 | 3.17 | 1.46 | 0.83 | 1.15 | 7.66 |
| Salicyluric Acid (SU) | 0.41 | 1.06 | 0.48 | 0.58 | 0.52 | 3.05 |
| Total Salicylic Acid (ST) | 3.05 | 6.60 | 3.67 | 2.60 | 4.21 | 20.13 |
| | | | Total Recovery = 48% | | | |
| | | | Excretion in % of Total Salicylic Acid (ST) | | | |
| Free Salicylic Acid (SA) | 34 | 48 | 40 | 32 | 27 | Av. 38 |
| Salicyluric Acid (SU) | 13 | 16 | 13 | 22 | 12 | Av. 15 |

[a] Urinary excretion of various salicylate fractions by four dogs receiving each twice daily 0.15 Gm./Kg. of sodium salicylate and 0.051 Gm./Kg. of sodium bicarbonate.

TABLE VIII[a]

| | 1st Day | 2nd Day | 3rd Day | 4th Day | 5th Day | Total |
|---|---|---|---|---|---|---|
| | | | Excretion in Gm. | | | |
| Free Salicylic Acid (SA) | 0.31 | 3.04 | 1.27 | 2.68 | 2.05 | 9.35 |
| Salicyluric Acid (SU) | 0.42 | 0.64 | 0.55 | 0.39 | 0.54 | 2.54 |
| Total Salicylic Acid (ST) | 1.51 | 7.68 | 3.22 | 6.36 | 5.98 | 24.75 |
| | | | Total Recovery = 59% | | | |
| | | | Excretion in % of Total Salicylic Acid (ST) | | | |
| Free Salicylic Acid (SA) | 20 | 40 | 39 | 42 | 34 | Av. 38 |
| Salicyluric Acid (SU) | 28 | 8 | 17 | 6 | 9 | Av. 10 |

[a] Urinary excretion of various salicylate fractions by four dogs receiving each twice daily 0.15 Gm./Kg. of sodium salicylate and 0.06 Gm./Kg. of magnesium trisilicate.

urinary excretion of SU was twice that of SA. It will require further investigations to determine whether this indicates a species difference or an alteration of the salicylate metabolism due to the inflammatory process.

## SUMMARY

Combinations of sodium salicylate with various antacids (sodium bicarbonate, aluminum hydroxide gel, magnesium trisilicate) and sodium or calcium ascorbate were investigated for toxicity and salicylate absorption and excretion. The test animals were rabbits and dogs.

The acute toxicity of sodium salicylate for rabbits seems to be reduced by sodium bicarbonate and by ascorbic acid in the form of its sodium and its calcium salt.

In rabbits the salicylate blood levels after a single dose are lower when an antacid is added except for large doses of sodium salicylate. The type of antacid is of relatively little importance.

When repeated doses of sodium salicylate are administered, the addition of magnesium trisilicate has no effect on the salicylate blood level, while sodium bicarbonate reduces it.

Data are presented showing the urinary excretion of free and conjugated salicylic acid in dogs. Similar data are given for the urinary excretion of free salicylic acid in rabbits. No salicyluric acid could be detected in rabbit urine.

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# An Unusual Impurity in Caffeine*

By HARRY M. BARNES, FRANKLIN KRAMER, and THOMAS R. WOOD

IN THE PURIFICATION of caffeine derived from plant sources the most difficult of the U.S.P. tests to pass is the test for "readily carbonizable substances." An inferior preparation gives a yellow to black color when dissolved in concentrated sulfuric acid, and this is generally interpreted as contamination with carbohydrate material. In the course of pilot plant operations on the refining of crude caffeine from green coffee, products were obtained which passed all of the U. S. P. tests except the carbonizable test, and they failed in this, not because of a yellow or black color, but because of a brilliant red color formed in the sulfuric acid solution. Repeated treatments of the caffeine in aqueous solution with charcoal failed to remove the contaminant completely.

It was finally found that the material responsible for the red coloration of the sulfuric acid could be separated by fractional crystallization from the caffeine solution, and its properties showed a great similarity to the "cafesterol" or cafestol isolated from the unsaponifiable fraction of green coffee oil by many previous workers (1–4).

The cafestol alone is almost completely insoluble in water, but it appeared to be soluble in caffeine solutions saturated at an elevated temperature. The caffeine would crystallize from this solution upon cooling to room temperature but, though not entirely free from the impurity, would leave the major portion of the cafestol in solution from which it would crystallize on long standing along with a small second crop of caffeine. From this mixture of caffeine and cafestol, the caffeine could be readily washed out with water, and the residue recrystallized from aqueous methanol.

The isolated material gave, in concentrated sulfuric acid, the same red color as the crude caffeine. The melting points of the isolated material and its derivatives are compared in Table I with the data of Slotta and Niesser (1), Wettstein (2), and Chakravorty (4). The agreement is taken as evidence of identity.

When dissolved in alcohol and treated with a drop of concentrated hydrochloric acid the isolated material gave a turquoise solution with a red fluorescence. This behavior is similar to that reported by Slotta and Niesser for crude cafesterol which they described as forming a rose color, rapidly changing to red then to violet, to blue and to greenish blue.

The solubility of the cafestol in the concentrated caffeine solutions is not unprecedented since Brock, Druckrey, and Hamperl (5) had found a greatly exalted solubility of 3,4-benzpyrene in aqueous solutions of caffeine and a lesser effect with theophylline, theobromine, and xanthine. Later Weil-Mailherbe (6, 7) studied the effect of many purines upon a large number of polycyclic aromatic hydrocarbons.

TABLE I.—COMPARISON OF MELTING POINTS

| | Crude Cafestol, ° | Acetate, ° | Cafestol-Maleic Anhydride Adduct, ° |
|---|---|---|---|
| Isolated | 154–155 | 159–162 | 191–191.5 |
| Slotta and Niesser | 155–157 | | |
| Wettstein | 153–155 | 157<br>168–170 (purified) | |
| Chakravorty | | | 190–192 |

The materials reported by Slotta and Niesser (1), by Wettstein (2), and by others (3, 4) were in all cases obtained by way of a saponification step. The material from which this present product was isolated was never subjected to conditions outside the *p*H range of 5 to 6 and was heated for only eight hours at 60–90°. Hence, it is reasonable to infer that the cafestol was present as such in the coffee and not as a saponifiable ester.

## EXPERIMENTAL

The caffeine which gave the deep red coloration with sulfuric acid was taken up in about 5 parts of water. The solution was heated to boiling to dissolve the caffeine, treated with Darco, boiled for about twenty minutes, filtered hot, and the solution allowed to crystallize by cooling to 30°. The first crop of caffeine was separated by filtration and the filtrate was allowed to stand overnight. A brown material separated out, was removed by filtration, and washed several times with cold water. After recrystallizing from methanol-water, it had a melting point of 154–155°.

* Received April 18, 1949, from Central Laboratories, General Foods Corporation, Hoboken, N. J.

It was acetylated by dissolving 100 mg. in 5 ml. of pyridine, adding 2 ml. of acetic anhydride, and allowing to stand overnight. The precipitate that formed when this solution was poured into 50 ml. of ice water was removed by filtration, and washed with water. After drying, its melting point was 159–162°. The acetate was converted to the maleic anhydride adduct by dissolving 36 mg. of the compound and 25 mg. of maleic anhydride in 1 ml. of C. P. benzene. The solution was held at 35° for thirty minutes and then allowed to stand at room temperature overnight. The crystals were separated by filtration, washed with benzene, and recrystallized from acetone by the addition of petroleum ether, melting point 191–191.5° with decomposition.

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# Viburnum Studies. XVI. Rate of Extraction of Uterine Sedative Potency*

By AARON B. SLOANE, ALBERT R. LATVEN, and JAMES C. MUNCH

**Samples of authentic Viburnum root bark as obtained commercially, a powder, a granulation, and a powdered extract prepared under commercial conditions, as well as a powdered extract prepared under laboratory conditions from this bark, were bioassayed on the isolated rat uterus for papaverine equivalent sedation. Solutions were then prepared by percolation from each of these barks and the samples assayed to determine uterine sedative potency.**

In connection with our investigation upon viburnum, the question arose (4) regarding the relative potencies of the crude viburnum bark and of powdered extract made therefrom. A number of samples were tested in which the powdered extract instead of being four times the strength of the crude drug, when tested on the isolated rat uterus against papaverine hydrochloride, was found to be even weaker than the crude viburnum bark (1, 2).

In order to obtain specific information on the effect of pharmaceutical manipulation upon physiological activity by the isolated rat uterus procedure, this study was made on four samples of authentic *Viburnum prunifolium* bark, identified by Dr. Heber W. Youngken. These samples included (1) whole root bark, lot no. 347; (2) powdered root bark, lot no. 349, prepared under commercial conditions from the whole root bark submitted for study; (3) granulation of the powdered root bark, lot no. 348, prepared under commercial conditions and submitted for inclusion in this study; (4) a powdered extract of this same root bark, lot no. 356, submitted as prepared under commercial conditions. These four samples were all obtained from the same lot of *Viburnum prunifolium* bark.

* Received September 1, 1948, from the Munch Research Laboratories, Inc., Upper Darby, Pa.
Presented to the Scientific Section, A. PH. A., San Francisco meeting, August, 1948.

## PROCEDURE

In preparation for the determinations of physiological potency by the method suggested in the National Formulary VII, the whole root bark was ground in a drug mill and sieved to medium-sized particles; the powdered and granulated root bark were used as submitted. Since the powdered extract was reported to be four times the crude drug strength, it was used as submitted but only one-fourth the quantity taken in the preparation of a percolate. Identical quantities of the whole root bark, the powdered root bark, and the granulation were weighed, and one-fourth that quantity of the powered extract; these were macerated with the N. F. menstruum of two parts of alcohol and one part of water, by volume, allowed to stand for a few minutes, then packed in a percolator and allowed to stand twenty-four hours. An excess of menstruum had been added to each percolator to insure extraction. Percolation was then made in the usual manner, so that 1 cc. of percolate was collected for every gram of the original crude drug represented. These four "fluidextracts" were then assayed at the same time on isolated rat uterine strips against papaverine hydrochloride to determine physiological potency. As shown in Table I it may be noted that the whole bark and the powdered root bark were of the same potency, indicating no loss in activity during the process of commercial powdering. The granulated material was 70% of the potency of the

TABLE I.—PHYSIOLOGICAL POTENCY OF VIBURNUM PRUNIFOLIUM—RATE OF EXTRACTION

| | Product | Papaverine Equivalent, γ/Gm. |
|---|---|---|
| (1) | Whole 347 | 500 |
| (2) | Powdered 349 | 500 |
| (3) | Granulated 348 | 350 |
| (4) | PE Commercial Preparation 356 | 1938 |

powdered or of the whole bark, indicating a significant loss in activity as a result of the steps of this process. The powdered extract of the root bark showed a potency substantially four times that of the crude bark, indicating no significant loss in potency as a result of such manipulations, with these lots of drugs. It appears that the steps taken in the preparation of this powdered extract were not responsible for any detectible loss in potency, although some steps in the preparation of the granulation had resulted in a significant loss in activity.

The marc in the percolator after collecting the first "fluidextract" from the crude powdered drug, lot no. 349 (sample no. 2), and the marc after collecting the corresponding solution from the powered extract (sample no. 4) were then re-extracted with additional volumes of the same N. F. alcohol-water menstruum, and successive lots of solution collected, each cc. corresponding to 1 Gm. of the original material weighed into the percolator. A total of 15 solutions on this type were collected. Assays on these indicated the results given in Table II. It may be noted

TABLE II.—RATE OF EXTRACTION OF PHYSIOLOGICAL POTENCY

| | Percolation of Crude Powdered VP 349 | | Percolation of PE of VP Root Bark 356 | |
|---|---|---|---|---|
| No. | γ/Gm. | % of Total | γ/Gm. | % of Total |
| 1 | 500 | 63 | 1938 | 60 |
| 2 | 140 | 18 | 665 | 20 |
| 3 | 125 | 16 | 500 | 15 |
| 4 | 25 | 3 | 150 | 5 |
| 5 | 0 | .. | 0 | .. |
| 6 | ... | .. | ... | .. |
| 7 | ... | .. | ... | .. |
| 8 | ... | .. | ... | .. |
| 9 | ... | .. | ... | .. |
| 10 | 0 | .. | ... | .. |
| 11 | ... | .. | 0 | .. |
| 12 | ... | .. | ... | .. |
| 13 | 0 | .. | 0 | .. |
| 14 | ... | .. | ... | .. |
| 15 | 0 | .. | ... | .. |

that the first fraction from the crude powdered viburnum contained 63% of the potency represented in the bark, whereas the corresponding fraction of powdered extract contained 60% or essentially the same proportions of the total potency extracted from the powdered extract. In other words, the rate of extraction of the crude drugs and of the powdered extract was identical in so far as the first sample was concerned.

As shown in Table II the corresponding sample of the crude drug and the powdered extract contained 18% and 20% in the second fraction; 16% and 15% in the third fraction; 3% and 5% in the fourth fraction; and subsequent samples showed no detectable potency by this method of assaying. These results suggest that the rate of extraction of potency is identical, using this method of percolation and this menstruum, on these samples of crude powdered viburnum marc and of powdered extract made therefrom under commercial conditions.

Because only 60% of the total physiological potency of these products was represented in the first fraction obtained as a "fluidextract," it would seem many of the bioassays on samples of viburnum have indicated potencies below the true value. The question also arises whether preparations prepared under commercial conditions by percolation using this method would show the same relative extraction; that is, whether commercial products are not completely exhausting the marc in connection with preparation of viburnum solutions. We have collaborative experiments with manufacturers under way in assaying the marc and various samples as obtained in successive fractions by percolation to obtain more information on this point. In the meantime the thought occurred to us that by shaking the sample of crude drug or powdered extract with the menstruum, in the same manner as used in the preparation of digitalis for bioassay in U. S. P. XIII, a more complete extraction might be obtained. We have started investigations on samples of these same lots of drugs, using the shaking process by comparison with the percolation process. Thus far results indicate that the shaking method has a more rapid rate of extraction of potency. If this is supported by commercial studies, a change in the method of preparation of liquid viburnum products might be considered, in the revision of the National Formulary, as well as in commercial practices.

## CONCLUSIONS

1. When assayed by the isolated rat uterus method against papaverine hydrochloride, whole authentic *Viburnum prunifolium* root bark and a powder made therefrom under commercial conditions showed the same potency.

2. A granulation from this powder under commercial conditions showed a significant loss in potency.

3. A powdered extract made from this sample under commercial conditions was approximately four times the potency of the crude drug, in agreement with expectations.

4. When crude drugs or the powdered extracts were extracted in a percolator using the N. F. VII alcohol-water menstruum, the rates of extraction were identical and all of the potency was obtained in the first four fractions.

5. Shaking appears to give more rapid extraction of physiological potency than percolation of viburnum bark.

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# Some New Procedures and Instruments Useful for Microbiological Antibiotic Testing by Diffusion Methods. I. A New Zone Reader*,†

By W. W. DAVIS, J. M. McGUIRE, and T. V. PARKE

**A new instrument having mechanical and optical provisions for the rapid and accurate reading of antibiotic inhibition zones is described and illustrated. Certain performance characteristics for the zone reader are presented.**

THE GREATER convenience of diffusion methods in comparison with serial or graded dilution methods for the microbiological assay of antibiotics leads to the general requirement of an accurate and rapid method of reading zone diamters. Various instruments and procedures have been adapted to accomplish this, prominent among them being the Quebec Colony Counter in which the colony counting grill is replaced with a millimeter scale, and the Balopticon which projects an enlarged image of the zone on a much enlarged scale. In both of these procedures the zone diameter is measured by bringing one edge of the zone or its projected image into coincidence with a zero reference line and then reading the position of the other edge or its projected image by interpolation between the lines of the magnified or enlarged millimeter scale. Still other devices have been suggested wherein other special features have been incorporated. To our knowledge, however, no instrument heretofore available combines all the desirable features for accurately and rapidly performing the reading of the great numbers of zones entailed in extensive assay programs.

A preliminary consideration of the usual relationship between potency and zone diameters (standard curve slopes) indicates that the accurate reading of zone diameters is a prerequisite to accurate assays. This can be illustrated by the case of Penicillin-G assayed by the porous pad method using a standard penicillin assay agar medium and *Staphylococcus aureus* as the test organism, wherein an error in the zone diameter of 0.1 mm. corresponds to an error of approximately 2.0% in the test result. In the assay of some other antibiotics the consequent error is even greater. It is then obviously desirable to measure zone diameters consistently to an accuracy of 0.1 mm. or better if this can be accomplished without serious sacrifice of speed. The present paper describes an instrument developed specifically to meet these requirements and presents some performance data.

## CONSTRUCTION

The essential provisions are (1) an optical system by means of which an enlarged image of the zone (or a part of it) possessing very high contrast between areas of growth and no-growth is projected onto a ground glass viewing surface bearing a straight lateral reference line; (2) a mechanical system for close and rapid alignment of the image of the zone edges with this reference line; (3) an enlarged scale for direct and accurate reading of the zone diameters; (4) a convenient dish guide for positioning the Petri dish, and for its rotation to bring successive zones into position for measurement; and (5) mechanical design which provides for efficiency of manual and visual operations.

The optical system is shown schematically in the drawing of Fig. 1. A horizontal beam of light from a concentrated filament source, $S$, is collimated by lens, $L_1$, and reflected into a vertical direction by a mirror, $M_1$. This beam then passes through a 1-inch diameter lens, $L_2$, set in the stage of the instrument, which focuses the beam to an image of the concentrated source at the position, $D$, where it passes through a circular opening of $^1/_8$ inch diameter. $D$ is located about 2 inches above the stage, being carried in an arm which extends forward for the

* Received Jan. 26, 1949, from the Lilly Research Laboratories, Indianapolis, Ind.
† It is a pleasure to acknowledge helpful discussion of statistical aspects of the problem with Mr. John C. Hansen, and many experiments arranged by Louise Niles to measure performance of the reader and of alternative methods of measurement.

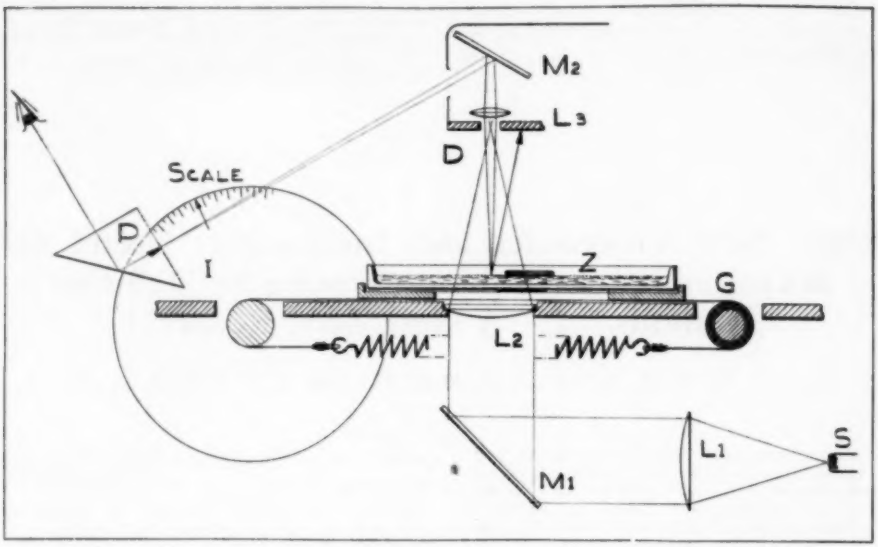


Fig. 1.—Diagram of the mechanical and optical systems of the antibiotic zone reader.

rear of the instrument. This arm also carries a lens, $L_1$, and a mirror, $M_2$.

The dish containing the agar medium with its inhibition zones is placed in the dish guide, $G$, on the stage with the beam passing upward through it. The lens, $L_2$, projects an image of that portion of the zone, $Z$, lying in the circular beam onto the ground glass surface at $I$. The mirror, $M_2$, is a first surface mirror so that a double image is avoided. A magnification of about 2 to 3 diameters is used. The depth of focus is such that the vertical distance $L_2$ to $Z$ is not critical, but $L_2$ is made vertically adjustable to provide for changes in the thickness of the Petri dish and the agar layer. The slight variations in thickness of agar which occur within an established routine do not result in loss of clear focus.

The diaphragm, $D$, is for the purpose of accentuating contrast in the image. This results from the exclusion of those rays which are scattered (refracted) by only a few degrees from their incident direction. This is a circular analogy of the schlieren effect commonly used for locating refractive index gradients. The projected image is observed through an opposed face of the prism, $P$.

The mechanical system for forward and backward movement of the dish consists of a belt carried by two pulleys at front and rear of the stage. The belt is discontinuous under the stage, the ends being attached to connecting springs to keep the belt taut about the two pulleys. Above the stage the belt rides almost without friction on the smooth surface of the stage. The dish guide is cemented on the belt and a rectangular opening 1 x 2 inches in both the dish guide and the belt passes the beam regardless of the forward or backward position of the dish guide.

The front pulley is a $^1/_2$-inch smooth stainless steel rod. The rear pulley is a rubber-covered rod to provide greater friction with the belt. The front pulley carries a large knob on each end. One is used for turning the pulley to move the belt forward or back and the other carries an enlarged scale, marked to show the movement of the belt from a zero position. A dead stop on the turning knob is made to coincide with zero position on the scale. When the knob is held against the dead stop with the scale zero at the index mark, turning the rear pulley with its enlarged knob causes the belt to slide over the smooth front pulley and its position can be adjusted easily so that one edge of an inhibition zone is brought to a position where its image is in contact with the straight reference line on the viewing surface. When the rear pulley is then left free and the front knob is turned, the belt follows the front pulley until the image of the opposite edge is brought into contact with the reference line. The millimeters of movement of the belt and dish are read off the enlarged scale.

Slippage of the belt was encountered in some of the earliest instruments as a result of friction in the rear pulley bearings. In the absence of such friction no slippage has been encountered.

The scale is enlarged about seven times over the movement of the belt. Thus, the smallest graduations showing 0.2 mm. of movement of the belt and Petri dish are about 1.4 mm. apart. It is very easy to read on the lines (even tenth-millimeters) or between the lines (odd tenth-millimeters). Whole millimeters are numbered over the range from 10 to 35 mm.

The dish guide is a thin circular plate with a flange inside of which the commonly used Petri dishes fit closely. In the center of the guide a small circle of a thin friction cloth is cemented to provide a brake to small unwanted movements of the dish on its guide. On the other hand this provides a pivot for easy rotation of the plate to bring successive zones into position for measurement.

The location of the viewing surface (on the rear

of the prism) reduces interference of illumination normally above and behind the line of sight of the operator. The distance from the eye to the image is very nearly the same as from the eye to the scale to facilitate the rapid and easy shift of vision without refocusing the eyes. This is an important factor in performance where readings are made over prolonged periods. The design presupposed the measurements to be made with the operator seated and with the instrument placed on a table of desk height. The commercially available model of the instrument is shown in Fig. 2.

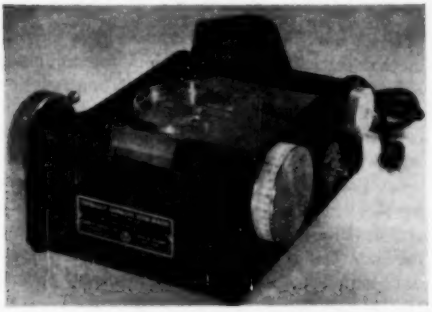

Fig. 2.—A commercially available model of the antibiotic zone reader, produced by Fisher Scientific Co., Pittsburgh, Pa.

Zone readers of the type described have been used exclusively, both in the research testing and in the production testing, in this company for over three years. This experience has proved the reader to be satisfactory both as to accuracy and speed of operation.

## PERFORMANCE

Performance of the instrument can be specified in terms of three factors: frequency distribution of readings as regards tenth-millimeter divisions, the reproducibility of rereading of zones, and average speed of reading. The contribution of the first two factors to over-all error is also considered briefly.

The first-mentioned aspect of performance is shown in Fig. 3. Using the Quebec Colony Counter the operators were instructed to read by interpolation between the millimeter scale marks to the nearest quarter millimeter. If this had been done consistently approximately 25% of the values would have ended in each 0.00, 0.25, 0.50, and 0.75. The resulting distribution is approximately what would have occurred if the operators read to the nearest quarter millimeter one-half the time and only to the nearest whole millimeter one-half the time.

Using the zone reader the operators were instructed to read to the nearest tenth millimeter (the odd tenths by interpolation between lines marking 0.0-, 0.2-, 0.4-, 0.6-, and 0.8-mm. endings). When this is done consistently approximately 10% of the readings end with each tenth millimeter from 0.0 to 0.9 mm. The lower part of the chart shows that readings were made consistently and without prejudice to the nearest 0.1 mm.

Such failure to read consistently by interpolation between millimeter lines does not show up in test results as a source of scattering of data, or inability to reproduce values by rereading. In fact, the reproducibility of rereading a large number of zones appears *better* when the coarser scale is used. In spite of this, large errors are introduced into small groups of data representing the assay of individual samples from the use of a coarse scale. If a 1.0-mm. scale were used consistently and accurately the error could be as large as 0.5 mm. resulting for a typical case in an error of assay of 12% from this cause. If a 0.5-mm. scale were consistently and

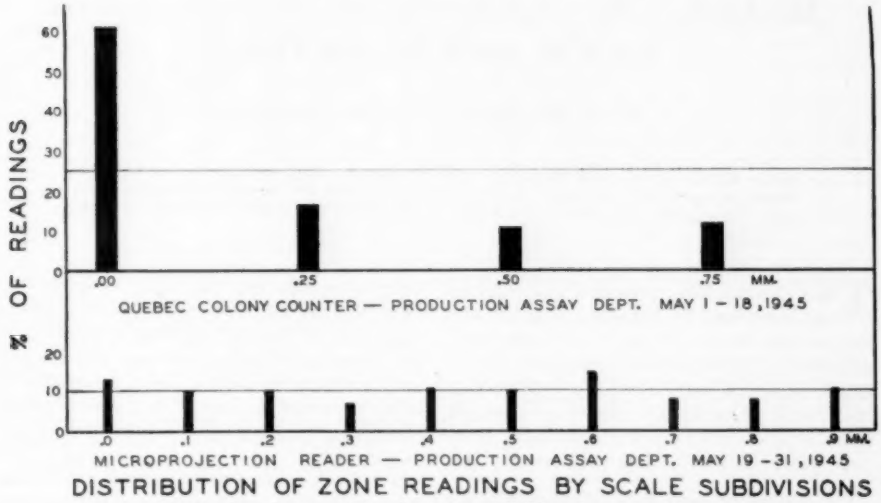


Fig. 3.—Distribution of zone readings by scale subdivisions for two reading procedures.

accurately used it would result in errors from this cause up to 6.0%. While such errors are not easy to locate against a background of other variables, they represent a real and significant contribution to over-all errors.

The lower chart shows that readings were taken with the zone reader without appreciable prejudice to 0.1 mm. The inherent error of reading consistently and accurately to 0.1 mm. contributes a maximum error of 1% in the typical example referred to above.

The reproducibility of rereading of zones becomes a significant measure of performance when the scale subdivision is comparable with the error of resetting the zone edge on the index mark. Two experiments were performed to determine the inherent error of measuring the zone diameters. In one experiment streptomycin zones with *Bacillus subtilis* were measured and in the other penicillin zones with *S. aureus*. In the first, the standard deviation of reading a zone was found to be 0.057 mm. based on repeated readings of three operators reading and rereading seven plates, each with four zones. In the second, using *S. aureus*, the standard deviation of measuring a zone was found to be 0.056 mm. In this experiment each of six operators read and reread six plates of four zones each. These two experiments indicate that the ability to reproduce the reading of a zone is such as to justify the use of a 0.1-mm. scale for the measurements.

Since the operations involved in the microbiological antibiotic testing routine are carried on successively, the entire reading operation on all plates occupies a period generally of less than two hours during the first part of the day. The practice of having one individual read the zone diameters and call off the results to another person who records them speeds up the operation. Such a team reads four zone plates at a rate of from 150 to 180 plates per hour, or from 600 to 720 zones per hour. In the case of one operator reading and recording diameters the rate has been found to be approximately one-half as great.

## SUMMARY

1. A new instrument is described, having mechanical and optical provisions for rapid and accurate reading of antibiotic inhibition zones.

2. Considerations are described which indicate the desirability of reading zone diameters with a scale as fine as 0.1 mm.

3. Some performance characteristics are given for this zone reader. No direct comparison with performance of other presently used reading methods has been made, but the performance data supplied allow this to be done by testing laboratories using other reading methods.

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# Some New Procedures and Instruments Useful for Microbiological Antibiotic Testing by Diffusion Methods. II. An Automatic Delivery Pipette for Use with Porous Disks*

By W. W. DAVIS and J. M. McGUIRE

**An automatic filling and emptying capillary pipette useful in applying water solutions of antibiotics to porous disks is described and its design illustrated. The use of this pipette increases the speed of operation in routine antibiotic testing.**

THE CYLINDER plate method of antibiotic assay introduced by Abraham, *et al.* (1) and Schmidt and Moyer (2) gives results largely independent of the volume of solution placed in the cups, within practical limits. For routine testing where great numbers of samples are involved, this has represented a decided advantage. However, other factors such as the generally less sharp zone edges resulting from the use of cups than from the use of porous disks, the occasional displacement of cups from their initial position, and imperfect contact of the cups with the agar surface with consequent imperfect or noncircular zones represent sources of comparative disadvantages in the use of cups compared to the use of porous disks.

Porous disks were first employed by Dowdy, Vincent, and Vincent as reported by Foster and

* Received Jan. 26, 1949, from the Lilly Research Laboratories, Indianapolis, Ind.

Woodruff (3). This procedure was further developed and refined by Loo, *et al.* (4). In contrast to the cup-plate method, the use of porous disks as employed by the latter workers requires the application of a constant volume of solution to the disks. The general practice where porous disks have replaced cylinders has been to deliver approximately 0.1 ml. from commercially available 0.1- or 0.2-ml. graduated pipettes. This requires delivery between two marks onto each disk. The process is relatively slow and incurs some irreproducibility and some systematic errors. These probably result from drainage errors which are generally different for the first and second 0.1-ml. delivered from a 0.2-ml. pipette.

With these considerations in mind it appeared desirable to develop an automatically filling and automatically delivering pipette which would deliver rapidly and accurately reproducible appropriate volumes of solution onto porous disks. The structural features of such a pipette now commercially available[1] are shown in the figure.

Fig. 1.—Structural features of the automatic capillary pipette.

## PROCEDURE

Operation of the pipette is as follows. The pipette is inserted into the appropriately diluted solution of antibiotic to a point below the bulb. The solution rapidly fills the pipette, rising by capillarity to the inner tip of the pipette. The pipette is removed, the end touched to a cloth with the pipette held at an angle to remove the drainage drop from the external surface. The tip is placed flat on the surface of the porous disk which has been placed on the agar surface. The solution is rapidly drawn out of the capillary by the greater capillarity of the disk.

The limiting useful volumes of such pipettes are between 0.080 and 0.095 ml. when applied to the paper disks[2] used in our studies. The disks begin, immediately upon being placed on the agar surface, to take up fluid from the agar by capillarity. The useful volume of the pipette is limited by the ability of the disk to draw out all the solution by capillarity. It is found that if the solution is applied promptly after placing the disk on the agar surface the maximum capacity of the disk to remove the solution from such a pipette is approximately 0.100 ml. This capacity decreases even below 0.095 ml. after a few minutes on the agar surface. The arbitrary upper limit of 0.095 ml. allows an adequate margin of volume. The spread of pipette volumes below this figure is kept minimal, a lower limit of 0.080 ml. being arbitrarily set.

When varying volumes from 0.080 ml. to 0.100 ml. of solution at 1 u./ml. of penicillin were applied to the pads the zone diameter changed approximately linearly with volume. Between these limits this results in a change in zone diameter of 0.06 mm. per 0.001 ml. In a typical experiment this 0.06 mm. difference in zone diameter would produce a 1.2% change in assay figure. This illustrates the desirability of reproducing the volumes delivered to less than plus or minus 0.001 ml.

The practice of each operator using one pipette for all solutions eliminates error of using pipettes of different volumes. This is efficiently done by applying all "standard" pads and solutions first in a set of three, four, or five plates. The pipette is then rinsed by refilling and re-emptying three times with the next test solution. After this all test sample pads and solutions are applied. The pipette is then rinsed again in the same way before applying the "standard" solution in the next set of Petri dishes. Thus a relatively small effort is used in the rinsing operations.

The standard deviation of volumes delivered from a given pipette, measured as weights of 1% phosphate buffer using an analytical balance weighing to 0.1 mg., was 0.000521 Gm. in two groups of 12 deliveries. For these two groups different operators and different pipettes were employed. This corresponds to a standard deviation of 0.03 mm. of zone diameter or of 0.6% in the assay figure in a representative experiment.

Five experienced operators and one new operator were timed without previous warning. The average speed was two four-zone Petri dishes or eight disks per minute, using the automatic capillary pipettes. This included the necessary intervening rinsing operations as described, but not other preparatory operations. Individual performance of experienced operators varied from twenty-eight to thirty-six seconds per plate, while a new operator used forty-four seconds per plate. This speed of operations involved in using these pipettes is then seen to be entirely adequate for routine antibiotic testing programs. Since the use of such pipettes and porous disks has completely replaced the use of other devices in our laboratories no fair comparison as to speed with alternative procedures can be presented in this report.

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[1] Available from Fisher Scientific Co., Pittsburgh, Pa.
[2] Schleicher and Schuell, No. 740-B, 1/2-inch analytical filter paper disks.

## SUMMARY

1. The design and operation of an automatic capillary pipette useful in applying aqueous solutions of antibiotics to porous disks is described.

2. It is shown that these pipettes offer the required accuracy and permit the speed of operations necessary in routine antibiotic testing.



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# Some New Procedures and Instruments Useful for Microbiological Antibiotic Testing by Diffusion Methods*

## III. The Use of Prepared Tables for Converting Zone Diameters to Potencies

By W. W. DAVIS, J. M. McGUIRE, and T. V. PARKE

**A method for the use of prepared tables in converting zone diameters to potencies in the evaluation of antibiotics is described. The calculations involved are developed and explained and a typical table is given.**

THE POROUS disk-plate method of antibiotic assay involves the measurement of the diameters of a number of inhibition zones for a dilution of a test solution and their comparison with the diameter of zones resulting from a solution of known potency formed on the same Petri dish. In practice either two "standards" and three "unknowns" or two "standards" and two "unknowns" have been generally used. The following procedure applies only when two "standards" of the same concentration and two "unknowns" of the same concentration are used.

### PROCEDURE

When the average diameter of the standard zones is subtracted from the average diameter of unknown zones on each dish, the difference is found to be approximately linear with the logarithm of the concentration of unknown. This relationship is generally assumed and used for the conversion of zone diameters to potency of the unknown. This relation can be expressed as

$$\log c_u = S(d_u - d_s),$$

when $c_u$ is units per ml. of unknown, $d_u$ is average diameter of the zone for unknown, $d_s$ is average diameter for the standard (1 u./ml.) solution, and $S$ is the slope of a standard curve, which varies somewhat from day to day and is determined by the preparation of plates with zones resulting from a series of known dilutions about 1 u./ml. The slope is the increase in zone diameter in millimeters found by extrapolating the curve of zone diameter vs. log concentration to a tenfold change in concentration.

Employing two "standard" zones of 1.0 u./ml. and two "unknown" zones, we have found the comparison of zone diameters and the conversion to potency of the unknown solutions to be expedited and rendered somewhat more accurate by the use of (1) sums and differences of diameters instead of averages and (2) conversion tables. These advantages can be fully exploited when using a calculating machine because of the greatly reduced number of operations.

Nine tables have been prepared, one for each $S$-value in steps of 0.5 over the range from $S = 8.0$ to $S = 12.0$, into which range all penicillin testing results have fallen. These tables are based on the relation

$$\log c_u = \frac{S}{2}(d_{u_1} + d_{u_2} - d_{s_1} - d_{s_2})$$

or

$$c_u = \text{antilog}\left[\frac{S}{2}(d_{u_1} + d_{u_2} - d_{s_1} - d_{s_2})\right]$$

* Received Jan. 26, 1949, from the Lilly Research Laboratories, Indianapolis, Ind.

TABLE I.—SLOPE 10

| | Δmm. | u./ml. | Δmm. | u./ml. | Δmm. | u./ml. | Δmm. | u./ml. | Δ mm. | u./ml. |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.0 | 1.00 | 2.0 | 1.26 | 4.0 | 1.58 | 6.0 | 2.00 | 8.0 | 2.51 |
| | 0.1 | 1.01 | 2.1 | 1.27 | 4.1 | 1.60 | 6.1 | 2.02 | 8.1 | 2.54 |
| | 0.2 | 1.02 | 2.2 | 1.29 | 4.2 | 1.62 | 6.2 | 2.04 | 8.2 | 2.57 |
| | 0.3 | 1.04 | 2.3 | 1.30 | 4.3 | 1.64 | 6.3 | 2.07 | 8.3 | 2.60 |
| | 0.4 | 1.05 | 2.4 | 1.32 | 4.4 | 1.66 | 6.4 | 2.09 | 8.4 | 2.63 |
| | 0.5 | 1.06 | 2.5 | 1.33 | 4.5 | 1.68 | 6.5 | 2.11 | 8.5 | 2.66 |
| | 0.6 | 1.07 | 2.6 | 1.35 | 4.6 | 1.70 | 6.6 | 2.14 | 8.6 | 2.69 |
| | 0.7 | 1.09 | 2.7 | 1.36 | 4.7 | 1.72 | 6.7 | 2.16 | 8.7 | 2.72 |
| | 0.8 | 1.10 | 2.8 | 1.38 | 4.8 | 1.74 | 6.8 | 2.19 | 8.8 | 2.75 |
| | 0.9 | 1.11 | 2.9 | 1.40 | 4.9 | 1.76 | 6.9 | 2.21 | 8.9 | 2.79 |
| Δmm. | 1.0 | 1.12 | 3.0 | 1.41 | 5.0 | 1.78 | 7.0 | 2.24 | 9.0 | 2.82 |
| (+) | 1.1 | 1.14 | 3.1 | 1.43 | 5.1 | 1.80 | 7.1 | 2.26 | 9.1 | 2.85 |
| | 1.2 | 1.15 | 3.2 | 1.45 | 5.2 | 1.82 | 7.2 | 2.29 | 9.2 | 2.88 |
| | 1.3 | 1.16 | 3.3 | 1.46 | 5.3 | 1.84 | 7.3 | 2.32 | 9.3 | 2.92 |
| | 1.4 | 1.17 | 3.4 | 1.48 | 5.4 | 1.86 | 7.4 | 2.34 | 9.4 | 2.95 |
| | 1.5 | 1.19 | 3.5 | 1.50 | 5.5 | 1.88 | 7.5 | 2.37 | 9.5 | 2.99 |
| | 1.6 | 1.20 | 3.6 | 1.51 | 5.6 | 1.91 | 7.6 | 2.40 | 9.6 | 3.02 |
| | 1.7 | 1.22 | 3.7 | 1.53 | 5.7 | 1.93 | 7.7 | 2.43 | 9.7 | 3.06 |
| | 1.8 | 1.23 | 3.8 | 1.55 | 5.8 | 1.95 | 7.8 | 2.46 | 9.8 | 3.09 |
| | 1.9 | 1.24 | 3.9 | 1.57 | 5.9 | 1.97 | 7.9 | 2.48 | 9.9 | 3.13 |
| | 0.0 | 1.00 | 2.0 | 0.79 | 4.0 | 0.63 | 6.0 | 0.50 | 8.0 | 0.40 |
| | 0.1 | 0.99 | 2.1 | 0.79 | 4.1 | 0.62 | 6.1 | 0.50 | 8.1 | 0.39 |
| | 0.2 | 0.98 | 2.2 | 0.78 | 4.2 | 0.62 | 6.2 | 0.49 | 8.2 | 0.39 |
| | 0.3 | 0.97 | 2.3 | 0.77 | 4.3 | 0.61 | 6.3 | 0.48 | 8.3 | 0.39 |
| | 0.4 | 0.95 | 2.4 | 0.76 | 4.4 | 0.60 | 6.4 | 0.48 | 8.4 | 0.38 |
| | 0.5 | 0.94 | 2.5 | 0.75 | 4.5 | 0.60 | 6.5 | 0.47 | 8.5 | 0.38 |
| | 0.6 | 0.93 | 2.6 | 0.74 | 4.6 | 0.59 | 6.6 | 0.47 | 8.6 | 0.37 |
| | 0.7 | 0.92 | 2.7 | 0.73 | 4.7 | 0.58 | 6.7 | 0.46 | 8.7 | 0.37 |
| | 0.8 | 0.91 | 2.8 | 0.73 | 4.8 | 0.58 | 6.8 | 0.46 | 8.8 | 0.36 |
| | 0.9 | 0.90 | 2.9 | 0.72 | 4.9 | 0.57 | 6.9 | 0.45 | 8.9 | 0.36 |
| Δmm. | 1.0 | 0.89 | 3.0 | 0.71 | 5.0 | 0.56 | 7.0 | 0.45 | 9.0 | 0.36 |
| (−) | 1.1 | 0.88 | 3.1 | 0.70 | 5.1 | 0.56 | 7.1 | 0.44 | 9.1 | 0.35 |
| | 1.2 | 0.87 | 3.2 | 0.69 | 5.2 | 0.55 | 7.2 | 0.44 | 9.2 | 0.35 |
| | 1.3 | 0.86 | 3.3 | 0.68 | 5.3 | 0.54 | 7.3 | 0.43 | 9.3 | 0.34 |
| | 1.4 | 0.85 | 3.4 | 0.68 | 5.4 | 0.54 | 7.4 | 0.43 | 9.4 | 0.34 |
| | 1.5 | 0.84 | 3.5 | 0.67 | 5.5 | 0.53 | 7.5 | 0.42 | 9.5 | 0.34 |
| | 1.6 | 0.83 | 3.6 | 0.66 | 5.6 | 0.53 | 7.6 | 0.42 | 9.6 | 0.33 |
| | 1.7 | 0.82 | 3.7 | 0.65 | 5.7 | 0.52 | 7.7 | 0.41 | 9.7 | 0.33 |
| | 1.8 | 0.81 | 3.8 | 0.65 | 5.8 | 0.51 | 7.8 | 0.41 | 9.8 | 0.32 |
| | 1.9 | 0.80 | 3.9 | 0.64 | 5.9 | 0.51 | 7.9 | 0.40 | 9.9 | 0.32 |

Each table includes both positive and negative values of the algebraic sum $(d_{u_1} + d_{u_2} - d_{s_1} - d_{s_2})$, or "Δ mm." The table to be used is chosen according to the $S$ value obtained from the standard curve for the day.

The algebraic sum, Δ mm., is given to the nearest 0.1 mm. and the resulting figure for u./ml. is given to the nearest 0.01 u./ml. The tables extend from −9.9 to +9.9 mm., the algebraic sum of diameters, corresponding to nearly a 5-mm. difference in *average* diameter of "standard" and "unknown" zones. This covers a range wider than the range of valid applicability of this linear relation for all $S$ values.

A set of such tables in ditto form is available free from these laboratories. The tabular arrangement is designed to promote speed and accuracy in the conversion of test results to potency. One of these tables, for $S = 10$, is reproduced here (see Table I). The ease and speed of using such a table is greatly dependent on the grouping and spacing of figures.

The use of these tables avoids the averaging or rounding-off steps in the conversion of zone diameters to u./ml. until the conversion has been effected to the nearest 0.01 u./ml. for each dish. It in no way reduces errors due to the failure of the assumed linear relation between log $c_u$ and zone diameters at low and high values of $c_u$.

In tests in which $c_s$ is other than 1.0 u./ml. the value of u./ml. given in the table is a ratio of $c_u$ to $c_s$, rather than absolute units per ml. In this case as before the tables are valid only over a range of validity of the assumed linear relation of log $c$ and zone diameter.

## SUMMARY

1. It is pointed out that where equal numbers of "standard" and "unknown" zones are used in each dish there are advantages in using sums and differences of zone diameters rather than average diameters.

2. A convenient table is illustrated by which such values are efficiently converted to antibiotic concentrations.

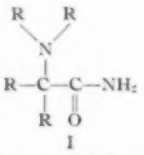
# Acetamide Derivatives*,†

By CHARLES O. WILSON‡

**Twelve derivatives of substituted aminoacetamides were prepared. The amides prepared and subjected to preliminary pharmacological investigation were all white, crystalline solids. In general, all were found to be nontoxic. No desirable anticonvulsant, antispasmodic, antihistaminic, or sedative activities were observed.**

ACETAMIDE derivatives have been found by numerous workers to possess sedative or analgesic properties. Many types of derivatives are reported in the literature especially those having aryl, alkyl, and halogen on the alpha carbon atom and/or aryl and alkyl groups on the nitrogen of the amide. An amide linkage can be considered as being present in some of the sedatives and anticonvulsants such as barbiturates and hydantoins. Acetamide derivatives having an amino group on the alpha carbon atom do not appear to have been studied pharmacologically.

In 1943, Henze (1) showed that the alkaline hydrolysis of 5,5-diphenylhydantoin yields alpha-aminodiphenylacetamide. This led Billman (2) to prepare a series of derivatives of alpha-aminodiphenylacetamide, including compounds that were substituted on the amide nitrogen and the amino group. The most active anticonvulsant was the unsubstituted alpha-aminodiphenylacetamide. It was, therefore, thought worth while to prepare compounds of type I for pharmacological testing.



I

R = hydrogen, phenyl, methyl ethyl, or butyl.

## EXPERIMENTAL[1]

**1. Dimethylaminoacetamide.**—Dimethyaminoacetonitrile (3) (10 Gm.), cooled in an ice bath, was mixed with a concentrated sulfuric acid (25 cc.), allowed to stand at room temperature for three days, poured on chipped ice, and carefully neutralized with ammonium hydroxide The solution was saturated with sodium chloride and extracted severa' times with chloroform. After combined chloroform extracts were dried with anhydrous sodium sulfate, they were evaporated to dryness. The yield of dimethylaminoacetamide 8.7 Gm. (72%); m. p. 95–96° (3).

**2. α-Aminopropionamide.**—Acetaldehyde (44 Gm.), ammonium chloride (59 Gm.), potassium cyanide (65 Gm.), and water (100 cc.) were shaken for a period of forty-eight hours at room temperature. The resultant reaction mixture was extracted four times with an equal volume of ether, dried with anhydrous sodium sulfate, and the ether removed by evaporation.

This crude α-aminopropionitrile was cooled to −10°, carefully mixed with an equal volume of concentrated sulfuric acid, allowed to stand three days at room temperature, poured on chipped ice, neutralized with ammonium hydroxide, saturated with sodium chloride, and extracted with chloroform. After the combined chloroform extracts were dried with anhydrous sodium sulfate, they were evaporated to dryness. The crude α-aminopropionamide was crystallized from chloroform; yield 54.5 Gm. (62%); m. p. 71–73° (5).

**3. α-Phenylaminopropionamide.**—α-Phenylaminopropionitrile (6) (10 Gm.) was mixed with cold concentrated sulfuric acid (25 cc.), allowed to stand at room temperature for two days, poured on chipped ice, neutralized with ammonium hydroxide, and saturated with salt. The insoluble α-phenylaminopropionamide precipitated and was collected on a Büchner funnel. It was recrystallized from alcohol; yield 8.1 Gm. (73%); m. p. 140–141° (6).

**4. α-Dimethylaminoisobutyramide.**—Dimethylamine hydrochloride (81.5 Gm.), sodium cyanide (49.0 Gm.), and acetone (93.6 cc.) were mixed in a suitable flask, shaken for two days at room temperature, extracted with ether; the combined ether extracts were dried with anhydrous potassium carbonate and the ether removed by evaporation.

The crude α-dimethylaminobutyronitrile (7–9) was added to an equal volume of cold concentrated sulfuric acid over a period of thirty minutes. This reaction mixture was allowed to stand forty-eight hours, poured on chipped ice, neutralized with ammonium hydroxide, saturated with salt, and extracted with ether. The combined ether extracts were dried with anhydrous sodium sulfate and the ether removed by evaporation. It was very difficult to recrystallize the free amide, and the hydrochloride was prepared by dissolving the amide in warm alcohol containing hydrogen chloride. On chilling, the α-dimethylaminoisobutyramide hydrochloride crystallized; yield 61.4 Gm. (37%) calculated on dimethylamine hydrochloride; m. p. 230–231°.

**5. α-Phenylaminoisobutyronitrile.**—Acetonecyanhydrin (10) was treated with freshly distilled aniline according to the methods of Jacobson (11) and Bucherer and Grolee (12).

**6. α-Phenylaminoisobutyramide.**—α-Phenylaminoisobutyronitrile (80 Gm.) was converted to the

* Received April 4, 1949, from the Research Laboratories, University of Texas, College of Pharmacy, Austin, Tex.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.
† The work reported in this paper was made possible through a grant from the Lederle Laboratories, Inc., Pearl River, N. Y.
‡ Professor, Pharmaceutical Chemistry, University of Texas, College of Pharmacy, Austin.
[1] All melting points are uncorrected.

amide by the same procedure that was used for α-dimethylaminobutyronitrile. It was extracted with ether and then was recrystallized from water; yield 72 Gm. (81%); m. p. 133–135° (10, 13).

**7. α-Amino-α-Phenylacetamide.**—α-Aminomandelonitrile (20 Gm.) was treated with sulfuric acid according to the method that was followed for α-dimethylaminoisobutyramide; then it was extracted with ether. The amide was recrystallized from benzene and alcohol; yield 14.7 Gm. (65%); m. p. 130–131° (14).

**8. α - Methylamino - α - Phenylacetamide.**—α - Methylamino-α-phenylacetonitrile was prepared from benzaldehyde (30 Gm.), sodium bisulfite (100 cc. saturated solution), methylamine (10 Gm.), and sodium cyanide (25 Gm.) by the method of Luten (15). The ether was removed by evaporation leaving the crude nitrile.

The impure α-methylamino-α-phenylacetonitrile was hydrolyzed to the amide by the procedure outlined for α-phenylaminopropionamide. It was recrystallized from water; yield 17.6 Gm. (38%); m. p. 153–155° (16).

as stated for α-diethylamino-α-phenylacetamide. Alcohol and water (4 to 1) was used to recrystallize the amide; yield 81.2 Gm. (31%); hydrochloride m. p. 203–204°.

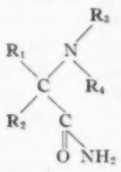
**12. α - Phenylamino - α - Phenylacetamide.**—α-Phenylamino-α-phenylacetonitrile was prepared by the method of Erdmann (17). This nitrile was treated with sulfuric acid in the same manner as was given for the preparation of α-diethylamino-α-phenylacetamide. The amide formed is recrystallized from water; yield 169.5 Gm. (75%); m. p. 122–123°.

**13. α-Phenyl α-Aminopropionamide.**—α-Phenyl-α-aminopropionitrile was prepared according to Jawelow (18) from acetophenone (120 Gm.) and converted to the amide by the procedure used for α-phenylaminopropionamide; yield 34.4 Gm. (21%); m. p. 249–250° (hydrochloride).

## DISCUSSION

The amides prepared and subjected to preliminary pharmacological investigation were all white, crystalline solids (see Table I). In general, all, too, were found to be nontoxic. No desirable anticonvulsant, antispasmodic, antihistaminic, or sedative activities were observed. In the rat, α-ethylamino-α-phenylacetamide in a dosage of 50 mg./Kg. showed some anticonvulsant property; in a dosage of 100 mg./Kg. α-di-*n*-butylamino-α-phenylacetamide had a sedative effect.

TABLE I



| | $R_1$ | $R_2$ | $R_3$ | $R_4$ | Empirical Formula | Yield, % | M. P., Found[a] | Carbon, % Calcd. | Carbon, % Found | Hydrogen, % Calcd. | Hydrogen, % Found |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1. | H | H | $CH_3$ | $CH_3$ | $C_5H_{10}ON_2$ | 72 | 95–96 | 47.05 | 46.80 | 9.80 | 9.71 |
| 2. | $CH_3$ | H | H | H | $C_5H_{10}ON_2$ | 62 | 71–73 | 40.90 | 40.72 | 9.09 | 8.82 |
| 3. | $CH_3$ | H | $C_6H_5$ | H | $C_{10}H_{12}ON_2$ | 73 | 140–141 | 65.85 | 65.73 | 7.31 | 7.25 |
| 4. | $CH_3$ | $CH_3$ | $CH_3$ | $CH_3$ | $C_6H_{14}ON_2$·HCl | 37 | 230–231 | 43.31 | 43.01 | 8.43 | 8.26 |
| 5. | $CH_3$ | $CH_3$ | $C_6H_5$ | H | $C_{12}H_{16}ON_2$ | 81 | 133–135 | 67.41 | 67.22 | 7.86 | 7.74 |
| 6. | $C_6H_5$ | H | H | H | $C_8H_8ON_2$ | 65 | 130–131 | 64.00 | 63.78 | 6.66 | 6.43 |
| 7. | $C_6H_5$ | H | $CH_3$ | H | $C_9H_{10}ON_2$ | 38 | 153–155 | 65.84 | 65.71 | 7.31 | 7.27 |
| 8. | $C_6H_5$ | H | $C_2H_5$ | H | $C_{10}H_{12}ON_2$ | 41 | 252–253[a] | 67.41 | 67.26 | 7.86 | 7.68 |
| 9. | $C_6H_5$ | H | $C_2H_5$ | $C_2H_5$ | $C_{12}H_{16}ON_2$ | 36 | 143–144 | 69.90 | 69.61 | 8.73 | 8.61 |
| 10. | $C_6H_5$ | H | $(CH_2)_4CH_2$ | $(CH_2)_4CH_2$ | $C_{16}H_{22}ON_2$ | 31 | 203–204[a] | 73.28 | 73.02 | 9.92 | 9.74 |
| 11. | $C_6H_5$ | H | $C_6H_5$ | H | $C_{14}H_{14}ON_2$ | 75 | 122–123 | 74.34 | 74.21 | 6.91 | 6.65 |
| 12. | $C_6H_5$ | $CH_3$ | H | H | $C_9H_{10}ON_2$ | 21 | 249–250[a] | 65.85 | 65.00 | 7.31 | 7.18 |

[a] Hydrochloride.

**9. α-Ethylamino-α-Phenylacetamide.**—The nitrile and amide were prepared by the method used for α-methylamino-α-phenylacetamide (15); yield 22 Gm. (41%); hydrochloride m. p. 252–253° (dec.).

**10. α-Diethylamino-α-Phenylacetamide.**—α-Diethylamino-α-phenylacetonitrile was prepared from benzaldehyde (106 Gm.) according to Luten (15). To cold concentrated sulfuric acid (25 cc.) cooled in an ice bath, was added the nitrile (23 Gm.) in small quantities, allowed to stand at room temperature for two days, and neutralized with saturated sodium carbonate solution. The amide precipitated and was collected on a Büchner funnel. It was recrystallized from water and alcohol (9 to 1); yield 74.1 (36%); m. p. 143–144°

**11. α-Di-*n*-Butylamino-α-Phenylacetamide.**—Luten's procedure (15) was employed to prepare the nitrile, and hydrolysis to the amide was carried out

## SUMMARY

1. Twelve derivatives of substituted α-aminoacetamide were prepared.

2. α-Di-*n*-butylamino-α-phenylacetamide and α-phenylamino-α-phenylacetamide are new derivatives of acetamide.

3. α-Di-*n*-butylamino-alpha-phenylacetamide

had a sedative effect on rats in a dosage of 100 mg./Kg.

4. α-Ethylamino-α-phenylacetamide had weak anticonvulsant activity.

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# A Proposed Method of Isolating Plant Constituents*

By GEORGE D. CURTS† and LOYD E. HARRIS‡

**It has been found that ethylene glycol monoethyl ether (Cellosolve) will extract all plant constituents that are normally extracted by the use of petroleum ether, ether, chloroform, and dehydrated alcohol employed in that order. A scheme of isolating plant constituents is presented involving their extraction with Cellosolve and fractional separation by the addition of water.**

IN THE COURSE of attempting to isolate specific compounds from plant material by the standard or so-called Dragendorff method, certain difficulties were encountered and problems were noted which were objectionable, such as the appearance of chlorophyll in all organic solvent extractions and the inordinate amount of time required. It was thought that a new method might be found.

A proposed approach to the problem was to select a solvent which would extract from the plant sample as many of the constituents as possible. The next step would be to take this solution and immediately attempt to precipitate pure substances from it.

Ethylene glycol monoethyl ether, sold under the trade name of Cellosolve by the Carbide and Carbon Chemicals Corporation of Charleston, W. Va., and hereinafter referred to as such, was selected as the solvent to be used because of its unusual solvent powers. Cellosolve was found to be capable of dissolving the materials soluble in petroleum ether, ether, chloroform, and alcohol, as well as many water-soluble substances. It therefore appeared to be a suitable solvent for this study.

Another favorable property of cellosolve is its complete miscibility with water. This suggested at once the possibility of causing material to precipitate from solution by the addition of water.

The miscibility of Cellosolve with all of the common solvents precluded the idea of extracting a Cellosolve solution with any other solvent. However, the extraction of a Cellosolve solution after having been diluted with water was postulated. It was found that a method is available for separating a Cellosolve-water mixture, that is, by extracting the mixture with chloroform and then evaporating the chloroform. The number of extractions necessary to complete this separation would surely separate the materials in solution into those more soluble in chloroform and those more soluble in water. This would be an advantage in a phytochemical study.

## EXPERIMENTAL

Studies were inaugurated to determine the feasibility of using Cellosolve as a plant extractant. In order to develop a scheme of resolution of a Cellosolve extract of a plant, a mixture of phlorizin, atropine, quinine, theophylline, and rosin (0.1 Gm. each), eucalyptus and soya oils (2 ml. each) was dissolved in 5 ml. of Cellosolve. This solution was used as a simulated Cellosolve extract of a plant, containing some of the usual types of constituents.

Water was added, drop by drop, to this simulated extract until a permanent cloudiness was obtained. This required approximately 0.25 ml. On standing, a 2-ml. layer came to the top. This was separated and shown to be soya oil by its index of refraction. Upon

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the addition of 3.5 ml. more of water to the solution, another persisting cloudiness was obtained. On standing, another 2-ml. portion of oil collected on top. This layer was shown to be eucalyptus oil by its odor and index of refraction. The addition of more water, in small portions, to the Cellosolve solution produced a cloudiness with each addition until a total of 75 ml. had been added, after which no further cloudiness could be obtained. The precipitate was shown to be rosin.

The resulting water-Cellosolve mixture was then extracted with chloroform. The aqueous layer remaining after this treatment was made alkaline with ammonia water and again extracted with chloroform. Atropine and quinine were shown to be present in both chloroform extracts, by precipitation reactions, but not in the residual water layer. Theophylline was found in the water layer. The presence of phlorizin in the aqueous layer and not in the chloroform extracts was established by its color reaction with ammonia.

From the data obtained thus far, a preliminary scheme of separation of constituents from a Cellosolve extract of a plant was developed (Fig. 1).

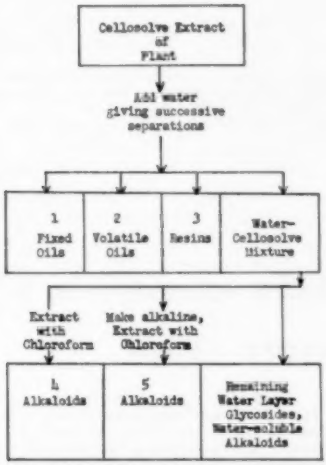


Fig. 1.—Preliminary separation scheme applied to known mixture.

This scheme was tested using plants having known constituents: *Malva rotundifolia*, L. (1), *Cephaelis ipecacuanha*, Brotero (2), and *Physalis mollis*, Nuttall (3). Cellosolve extractions of these plants were made using a Soxhlet-type extractor at reduced pressure so that the Cellosolve boiled at 55–60°. The marcs were then extracted with selected solvents to determine if additional materials could be obtained. Table I gives a compilation of the results.

The resins obtained by alcoholic extraction were soluble in Cellosolve, and the other materials not extracted by Cellosolve but by alcohol were water soluble.

TABLE I.—MATERIALS OBTAINED AFTER CELLOSOLVE EXTRACTIONS

| Solvent | Malva | Ipecac | Physalis |
|---|---|---|---|
| Petroleum ether | Nothing | Nothing | Nothing |
| Ether | Nothing | Nothing | Nothing |
| Chloroform | Nothing | Nothing | Nothing |
| Alcohol | Potassium nitrate | Carbohydrate, Resin | Potassium nitrate, Carbohydrate, Resin |

The Cellosolve extracts were concentrated under reduced pressure at 55–60° and studied according to the suggested scheme. Water was added in small portions and the resulting precipitates removed. The first material to be precipitated was not completely separated from solution until other materials had begun to appear. It was observed thereby that pure materials were not thrown out of solution by the addition of water in the clear-cut manner of the simulated extract. The combined material precipitated by water was resolved in a manner similar to that used for a petroleum ether extract of a plant sample. The types of compounds found in this fraction were: from Malva—wax, fatty oil, sterol, resin; from Ipecac—fatty oil, wax, resin; from Physalis—fatty oil, chlorophyll, tarry resin.

In making the chloroform extracts of the water-Cellosolve mixtures, after several extractions there appeared material floating at the interface of the water and chloroform layers. It was deduced that this was material which was insoluble in both water and chloroform and that the Cellosolve on being partitioned between the two layers was insufficient in either to hold the material in solution. This precipitate was added to the alcohol-soluble material obtained in the next step. The chloroform extract was evaporated to remove the solvents. The residue was then incompletely soluble in chloroform, and after it had been thoroughly extracted with chloroform, was dissolved in alcohol. Alkaloids were found in the chloroform and alkaline chloroform extracts of Ipecac and in the chloroform extract from Physalis. Malva contains no alkaloids. Resins were found in the chloroform extracts but not in the alkaline chloroform extracts. The chloroform-insoluble but alcohol-soluble materials were resins.

In the remaining water layers the following types of compounds were found: from Ipecac—alkaloids, glycosides, tannin, organic acids; from Physalis—an organic acid.

The scheme of separation, as shown in Fig. 2, incorporates certain modifications from the preliminary scheme which were found necessary during the studies upon the real plant extracts. Figure 2 also summarizes the materials which have been experimentally found in the fractions listed.

## CONCLUSIONS

1. Within the limits of practicality, Cellosolve will extract from a plant sample all of the constituents which may be obtained by petroleum ether, ether, chloroform, and alcohol.

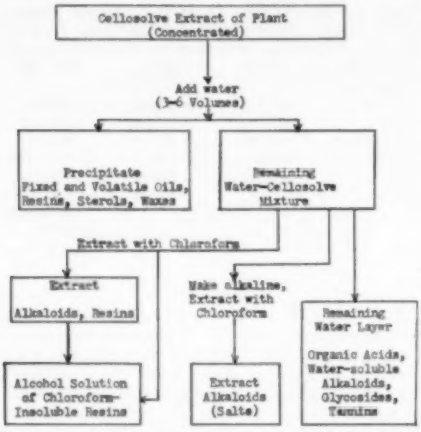
Cellosolve Extract of Plant (Concentrated)
Add water (3–6 Volumes)
Precipitate Fixed and Volatile Oils, Resins, Sterols, Waxes
Remaining Water–Cellosolve Mixture
Extract with Chloroform
Extract Alkaloids, Resins
Make alkaline, Extract with Chloroform
Remaining Water Layer
Organic Acids, Water-soluble Alkaloids, Glycosides, Tannins
Alcohol Solution of Chloroform-Insoluble Resins
Extract Alkaloids (Salts)

Fig. 2.—Final separation scheme applied to plant material.

2. The materials which are extracted by alcohol and not by Cellosolve are water soluble and may be obtained by aqueous extraction.

3. It is estimated that the extraction and separation of plant constituents using the Cellosolve method can be accomplished in about one-third the time required for the traditional four-solvent method.

4. The Cellosolve method entails only about one-half the cost of the four-solvent method, this estimate being based on present prices.

5. The necessity of conducting a continuous Cellosolve extraction at reduced pressure in order to keep the temperature at a reasonably low point is not considered to be an objection to the use of this method.

6. The slight residue remaining after evaporation of a sample of Cellosolve does not necessarily indicate dissolved extractive. Different volumes of a redistilled sample left about the same amount of this residue, which feels greasy or waxy. An investigator should acquaint himself with this phenomenon before using this solvent as an extractant.

7. Materials have been found to separate from Cellosolve by the addition of petroleum ether. Although this function was not utilized in the scheme of separation as proposed, it may be of value in specific cases.

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(3) Harris, Loyd E., THIS JOURNAL, **37**, 145(1948).

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# A Phytochemical Study of *Malva rotundifolia*, L.*

By GEORGE D. CURTS† and LOYD E. HARRIS‡

***Malva rotundifolia*, a plant which has for many years been used empirically as a drug, has been subjected to a systematic study. Constituents isolated, characterized, and reported include a fatty oil containing oleic, stearic, and palmitic acids, a wax that was primarily octacosane, a phytosterolin, arabinose, a mucilage, potassium nitrate, and potassium chloride. Alkaloids were shown to be absent.**

MANY PLANTS which have been used empirically as medicines by the peoples of the world have never been made the object of any systematic scientific study. A phytochemical study of *Malva rotundifolia* was initiated as a result of the reported household use of this plant in the treatment of glycosuria.

Malva has been used since ancient times as an emmenagogue (1). It is used today in the treatment of children's colic, indigestion, and intestinal inflammation (2). Malva has been used by a diabetic person who claimed that drinking the decoction caused the sugar in his urine to disappear (3). He had been told of its alleged value for this purpose by others. However, blood samples taken from this man, and from another

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diabetic, showed the same sugar content before, during, and after a two months' treatment with the decoction (4).

Treatises on materia medica classify Malva with the drugs having emollient, antiphlogistic, and other properties attributable to the mucilage content. Malva leaves, from *M. sylvestris* and *M. rotundifolia*, were included in the National Formularies IV and V as a component of Species Emollientes.

*Malva rotundifolia*, L. is one of the common species of the genus Malva, family *Malvaceae*, order *Malvales*. It grows wild in Europe and western Asia. Introduced into North America, it has escaped cultivation and is common throughout the temperate areas and Mexico. *M. rotundifolia* has procumbent stems spreading from a strong deep root, cordate leaves, long leaf stalks, small pale blue to purple flowers, and circular united carpels that appear like cheeseheads. Common names for the plant include: Blue-, Running-, Common-, or Low-Mallow; Cheese Plant; and Shirt Button Plant (1, 2, 5, 6).

The chemistry of the Malvas has not been thoroughly elaborated. Callegari, *et al.*, reported the presence of vitamins A, $B_1$, and C in amounts sufficient to prevent avitaminoses, their reports being based on feeding experiments with *M. sylvestris*. They also reported the presence of 1.06% mucilage (7). Malva Leaves, N. F. V., had a specification of not more than 16% ash. The coloring matter of the blooms of Malva is malvin, a very widely distributed 3-5 diglucoside (8). The fruit is reported to contain a bitter substance (1). The leaves are also reported to contain tannin (8).

## EXPERIMENTAL

A sample of Malva was identified as *Malva rotundifolia*, L. by Dr. Clyde H. Jones of the Botany Department, the Ohio State University. The sample contained stems, stalks, leaves, and fruits. The main bulk of the sample consisted of leaves. A quantity of the air-dried plant sample was ground in a Wiley Drug Mill through a No. 20-mesh screen. Table I gives the gross constitution of this sample.

**Extraction with Selective Solvents.**—Two samples of 25 Gm. each were extracted successively with selective solvents using Soxhlet extractors. Each extract was evaporated to remove the solvent and the residue weighed. Table II shows the percentages of extractive material obtained by each solvent. Percentages are not corrected for moisture content.

**Petroleum Ether Extract.**—This extract was evaporated to a greenish black semifluid mass. Shaking with water and 0.1 *N* hydrochloric acid dissolved nothing which would give a precipitate with phosphomolybdic acid or leave any residue upon evaporation. After washing the mass with warm acetone, a black tar-like material remained which was also insoluble in chloroform, alcohol, and petroleum ether. When the acetone solution was cooled, a green wax precipitated. This was purified to a white wax by precipitation from hot alcohol and acetone, yielding 0.039% from the original sample.

After five other methods had failed, the wax was resolved into different components by fractional crystallization from petroleum ether. The main bulk of the wax was shown to be a pure hydrocarbon, octacosane: m. p. 61.5°; carbon 84.3%; hydrogen 14.4%.

The acetone solution from which the wax had been collected was evaporated to an oleoresin which had the odor of fatty oils. Some of the customary characteristics of the oil were determined. These are shown in Table III. No material similar to the unsaponifiable matter could be isolated from the iodination residues, indicating that this material is attacked by the iodinating agent and thus contributes to the iodine value. Glycerin was identified in the aqueous residues from the saponification-number determinations, by the evolution of acrolein fumes upon concentrating and boiling the residue.

The fatty acids obtained from the saponification experiments were fractionated by the low-temperature method (9). The fractions were then brominated in chilled ether. No ether- or petroleum ether-insoluble bromides were found, showing the absence of acids with more than one double bond in

TABLE I

| Determination | Results, % | Method |
|---|---|---|
| Moisture | 7.0–7.4 | U. S. P. Toluene |
| Ash | 17.25–17.45 | U. S. P. |
| Kjeldahl Nitrogen | 0.405–0.42 | Hengar semimicro |
| Protein | 2.43–2.53 | Calculated |
| Water-soluble Extractive | 40.0 | Repeated hot water extractions |

TABLE II

| Solvent | Sample 1, % | Sample 2, % |
|---|---|---|
| Petroleum Ether | 1.25 | 1.20 |
| Ether | 0.77 | 0.71 |
| Chloroform | 1.46 | 1.66 |
| Alcohol | 7.44 | 7.83 |
| Totals | 10.92 | 11.40 |

TABLE III.—CHEMICAL CONSTANTS OF THE OIL

| | Sample 1 | Sample 2 |
|---|---|---|
| Iodine Value (Hanus) | 108. | 111. |
| Acid Value | 43.6 | 41.7 |
| Ester Value (by Difference) | 79.7 | 80.5 |
| Saponification Number | 123.3 | 122.5 |
| Unsaponifiable Matter | 51.7% | 50.4% |

the molecule. Comparison of the petroleum ether-soluble portions showed that the separation of saturated and unsaturated acids had not been complete. In each of these portions bromination derivatives were found by elemental analysis, indicating the presence of a mono-ethenoid acid such as oleic. Melting-point studies indicated the presence of stearic and palmitic acids in both portions.

| | Melting Point | Solidification | |
|---|---|---|---|
| Fraction Insoluble at −20° | 55–56° | 50–52° | |
| Fraction Insoluble at −48° | 56–57° | 52° | |
| Stearic (70%) and Palmitic Mixed | 55.8° | 54.5° | (9) |

**Chloroform Extract.**—The chloroform extract was evaporated to dryness. Hot water washings of the residue gave no precipitates with Mayer's reagent, picric acid, or Wagner's reagent. Alkaloids were thus shown to be absent. The water-insoluble portion was washed with acetone and a waxy residue remained. The acetone solution left a black tar upon evaporation.

The waxy residue was dissolved in hot alcohol. On cooling, a nearly white, flocculent precipitate, insoluble in petroleum ether, was obtained. It was recrystallized from alcohol and thoroughly washed with petroleum ether. It gave positive Salkowski and Liebermann-Burchard reactions, confirming that it was a sterol. When heated, it discolored at 232° and finally became a reddish melt at 237°. This decomposition at a fairly high melting point suggested that it is a phytosterolin. A portion of the sample was heated with 6% hydrochloric acid in amyl alcohol for forty minutes. The resulting brown solution was diluted with water and extracted with chloroform. The aqueous solution gave a positive Molisch reaction showing the presence of a carbohydrate residue after hydrolysis and substantiating, therefore, that the material is a phytosterolin.

The free sterol was obtained from the chloroform solution by evaporating the solvent and redissolving the residue in hot alcohol. The alcohol solution was filtered and evaporated. The free sterol was washed with petroleum ether after which it was found to have a melting point of 138–138.5°. The acetate was formed from the phytosterolin using acetic anhydride and it had a melting range of 144.5–146.5°.[1]

**Alcohol Extract.**—The alcohol extract was allowed to evaporate, giving, successively, a green precipitate, a brown precipitate, crystals, and a tarry resin. A solution of the green precipitate showed the typical fluorescence of chlorophyll. The brown precipitate and the resin were not characterized. The crystals were identified as potassium nitrate and potassium chloride. Alkaloids were shown to be absent from the alcohol extract by testing with Mayer's reagent, picric acid, and phosphomolybdic acid.

**Water-Soluble Constituents.**—A hot water extract of Malva was made by boiling a portion of the unpowdered sample with water, straining, and repeating the process a second time. The water extract was divided and placed in 18-inch sections of 4-inch Visking cellulose tubing and dialyzed with stirring in both inner and outer chambers for ninety-six hours, replacing the outer water five times.

The dialysate was concentrated under reduced pressure at 60–70°. During this concentration a brown, muddy precipitate formed. This precipitate was not completely combustible, leaving a white ash. A solution of this ash gave positive tests for calcium sulfate. The xanthoproteic reaction and the development of humin showed that the organic material precipitated from the dialysate was protein.

To remove protein material, the remaining solution of the dialysate was maintained at 60° for several days. The precipitate which formed was removed. Percolation of the concentrated, protein-free solution through activated charcoal removed the red color, leaving a pale yellow solution. This gave positive Tauber's and Disch's reactions for pentoses. Arabinose was shown to be present by the formation of arabinosazone.

The non-dialyzed portion was diluted with 5 volumes of isopropyl alcohol, whereupon a precipitate was produced. Since protein material was indicated by a test with phosphomolybdic acid, a solution of the precipitate in water was heated at 60° for forty-eight hours and the coagulate which formed was removed by filtration. The remaining solution still gave a positive Molisch reaction for carbohydrates but no precipitate with phosphomolybdic acid.

Since protein is essentially unchanged by denaturation, if the calcium were bound to it the calcium would have been precipitated by this coagulation. However, the remaining solution gave positive test for calcium, showing it to be bound to the carbohydrate. This carbohydrate can be called a mucilage since it is gel-like, nondialyzable, and cloudy, apparently colloidal, in solution. The mucilage is therefore a calcium salt. Furthermore, it may have an acetic acid fragment and a pentosan structure, since an acid hydrolysate gave fumes with the odor of acetic acid and positive Bial's and Disch's reactions for pentoses.

## SUMMARY

The materials isolated from *M. rotundifolia* were: fatty oil containing oleic, stearic, and palmitic acids; wax, primarily octacosane; a phytosterolin; potassium nitrate, potassium chloride, calcium sulfate; arabinose; mucilage; resins and tars. Alkaloids were shown to be absent. Composition determinations include: 7.2% moisture, 17.35% ash, 0.41% Kjeldahl nitrogen, 2.5% calculated protein, 40% water-soluble extractive.

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# The Chemical Assay of Aureomycin*

By JOSEPH LEVINE, EDWARD A. GARLOCK, JR., and HENRY FISCHBACH

**A fluorometric method and a colorimetric method are presented for the quantitative determination of aureomycin. Both procedures are simple and require only common reagents. Time rate studies are included for the separate steps in the procedures. In addition, the results obtained by the chemical methods are compared with those found by the cup-plate bioassay. The products used in this study were commercial samples of aureomycin in the dosage forms of powders, capsules, and troches.**

SINCE the advent of aureomycin, several types of bioassay techniques have been devised for the assay of the antibiotic (1, 2). Thus far, no chemical procedure for this purpose has appeared in the literature.[1] This paper presents two simple physicochemical methods for assaying aureomycin: a fluorometric procedure measuring the fluorescence developed by alkaline treatment, and a colorimetric procedure measuring the intensity of the color developed by acid treatment of aureomycin.

It has been shown that the antibiotic activity of aureomycin is rapidly lost in alkaline solution (3, 4). During the investigations in the laboratory it was observed that with progressive alkaline degradation, there occurs a concomitant fluorescence of increasing intensity. The rate at which the fluorescence is developed and the intensity of the maximum fluorescence are dependent upon the *p*H of the solution. In very weakly alkaline solutions the rate of development of the fluorescence is slow enough to be measured, and increases with temperature. From these considerations the fluorometric procedure was developed.

It has been demonstrated (5) that aureomycin exhibits several absorption maxima in the ultra violet region both in acid and alkaline solution. It was noted in this laboratory that the antibiotic develops a characteristic absorption maximum in the visible portion of the spectrum when treated with mineral acid. The rate at which the color develops is dependent upon temperature and acid concentration. These observations led to the colorimetric determination of aureomycin.

## FLUOROMETRIC METHOD

Since both the rate of development of fluorescence and the intensity of the fluorescence are dependent upon *p*H, it is necessary that the alkaline solution used to bring about the conversion be highly buffered and that it be at a *p*H at which the rate of conversion is sufficiently slow, at room temperature, to permit measurement of the initial, or blank, fluorescence. Such a reagent was prepared by mixing eight volumes of 20% (*w/v*) dibasic potassium phosphate solution with one volume of 20% (*w/v*) monobasic potassium phosphate solution. The *p*H of this solution is 7.6; in a dilution of 1 ml. of the reagent with 5 ml. of water (the proportions used in the procedure) the solution has a *p*H of 7.5. The rate of increase of fluorescence of aureomycin at this *p*H at room temperature (approximately 30°) is very slow. At 65° a maximum is reached in twenty minutes, while at 100° the maximum is reached within three minutes (Table I).

TABLE I.—RATE OF DEVELOPMENT OF FLUORESCENCE

| Time of Heating at 100°, Min. | Fluorescence Scale Reading |
|---|---|
| 1 | 19.0 |
| 2 | 34.5 |
| 3 | 35.0 |
| 4 | 35.0 |
| 5 | 35.0 |

Increasing the heating time up to thirty minutes has no effect. The developed fluorescence is quite stable for several hours (Table II); at higher concentrations the decrease is more rapid than at lower concentrations.

Measurements of fluorescence were made on a Coleman Electronic Photofluorometer Model 12, equipped with Filter B1 for the incident light and Filter B2 for the emitted fluorescence. Matched 18- x 150-mm. Pyrex test tubes were used as cells. For a fluorometric standard, a stock solution was prepared by dissolving 20.0 mg. of U. S. P. Quinine

* Received July 19, 1949, from the Federal Security Agency, Food and Drug Administration, Washington, D. C.
[1] The authors have been informed (personal communication) that similar methods were contained in a paper entitled "The Assay of Aureomycin by Instrumental Methods" by Homer A. Kelsey and Leon Goldman read by title only at the April, 1949, Symposium of the Antibiotics Study Section, National Institute of Health.

TABLE II.—STABILITY OF FLUORESCENCE

| Time After Heating, Min. | Fluorescence Scale Reading |
|---|---|
| 15 | 33.2 |
| 30 | 33.2 |
| 60 | 33.0 |
| 120 | 32.8 |
| 20 hours | 31.9 |

Sulfate in 1000 ml. of 0.1 *N* sulfuric acid; 5 ml. of this stock solution was diluted to 200 ml. with 0.1 *N* sulfuric acid. The resultant fluorometric standard is stored in the dark.

**Procedure.**—Set the fluorometer reading against the quinine sulfate fluorometric standard to a scale reading of 35.0. To 10 ml. of solution containing 2.5 to 35 mcg. of aureomycin add 2.0 ml. of the alkaline reagent. Stir, and read the fluorescence immediately. Heat in a steam bath or a boiling water bath for five minutes. Cool; read the fluorescence in from fifteen minutes to one hour. Subtract the initial fluorescence reading. Calculate the aureomycin content by reference to a standard curve prepared by treating suitable aliquots of a standard solution of aureomycin hydrochloride as described above.

Figure 1 is such a curve prepared from a solution containing 3.24 mcg. of pure aureomycin hydrochloride[1] per ml.; aliquots of from 1 to 10 ml. in 1.0-ml. steps were diluted to 10 ml., in duplicate. The linear relationship and the reproducibility are shown in the figure. An average reading of 2.20 scale units per mcg., with a standard deviation of ±0.04, was obtained.

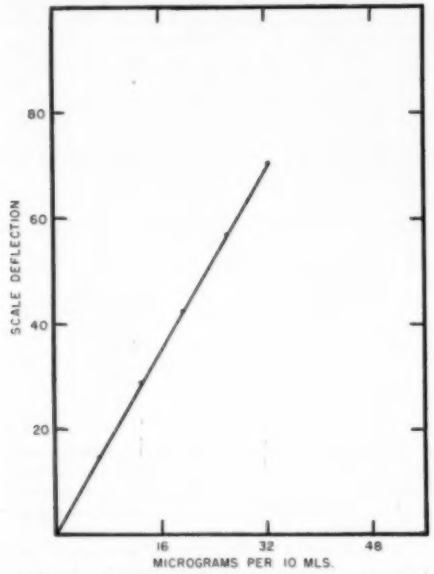


Fig. 1.—Standard curve showing the relation of aureomycin concentration to intensity of fluorescence.

[1] Obtained from Lederle Laboratories Division, American Cyanamid Co.

## COLORIMETRIC METHOD

Aureomycin, when heated with hydrochloric acid, develops a stable yellow color. The absorption curve obtained (Fig. 2) shows a characteristic absorption maximum at 445 mμ, while the curve obtained with untreated aureomycin shows minimum absorption at this wave length. The optical density of the developed color is proportional to the concentration of aureomycin, in accord with Beer's law (Fig. 3).

**Procedure.**—Prepare an aqueous solution of 1.0 mg. per ml. of the sample to be assayed. Transfer two 1.0-ml. aliquots to each of two 50-ml. volumetric flasks. Add 5.0 ml. of 2 *N* hydrochloric acid to one flask and 5.0 ml. of distilled water to the other flask as a control blank. Heat both flasks on a boiling water bath for five minutes, then cool the flasks under tap water, add 5.0 ml. of 2 *N* hydrochloric acid to the control blank and immediately make up with distilled water to the mark of both flasks. Transfer the solutions to 1.0-cm. colorimetric cells; set the photoelectric colorimeter at 100% transmittance for the blank using a 440-mμ filter. Replace this with the cell containing the unknown and read the per cent transmittance. Determine the concentration of the unknown solution by reference to a standard curve (Fig. 3) prepared by treating appropriate aliquots of a standard solution containing 1.0 mg. per ml. of pure aureomycin hydrochloride as described above.

Prior to fixing the conditions for assay by this procedure, consideration was given to the effect of acid concentration, time of heating, and stability of the developed color. It is evident from Table III

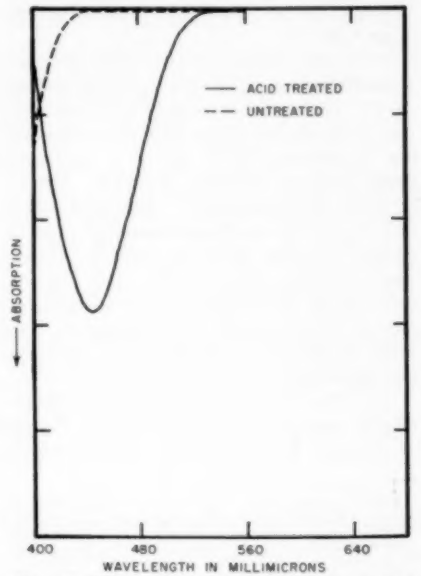


Fig. 2.—Recorded absorption curves of acid treated and untreated aureomycin solutions.

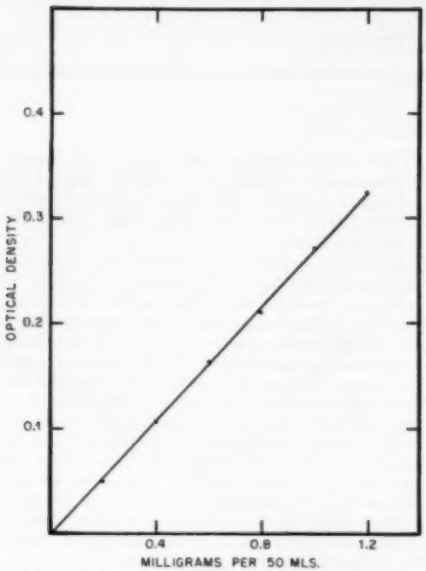


Fig. 3.—Standard curve showing the relation of aureomycin concentration to optical density using a 440 mμ filter.

that three minutes is adequate for the maximum color development. Optimum results are obtained by the use of 2 *N* hydrochloric acid, as shown in Table IV. Adequate stability of the developed color over a three-hour period is demonstrated in Table V.

Several experiments were conducted to determine the extent to which degradation products of aureomycin interfere with the assay procedures. A solution of aureomycin was oxidized by heating with potassium permanganate and phosphoric acid. After destruction of the excess permanganate with bisulfite, the solution was assayed by the colorimetric method. A negligible amount of color developed. Aureomycin solutions treated with acid according to the colorimetric method do not develop a fluorescence when made alkaline, while solutions treated with alkali according to the fluorometric method develop a negligible color when subjected to the colorimetric assay.

TABLE III

| Time of Heating, Min. | % Transmission |
|---|---|
| 1 | 73.2 |
| 2 | 60.3 |
| 3 | 53.3 |
| 5 | 53.6 |
| 7 | 53.3 |
| 10 | 53.7 |

TABLE IV.—EFFECT OF ACID CONCENTRATION (5-MINUTE HEATING)

| HCl Concentration | % Transmission |
|---|---|
| 0.1 *N* | 89.0 |
| 1.0 *N* | 55.1 |
| 2.0 *N* | 54.7 |
| 4.0 *N* | 54.7 |

TABLE V.—STABILITY OF COLOR

| Time of Standing, Hr. | % Transmission |
|---|---|
| 0.5 | 52.3 |
| 1.0 | 52.7 |
| 1.5 | 52.6 |
| 2.0 | 52.6 |
| 2.5 | 52.7 |
| 3.0 | 52.9 |

TABLE VI.—ASSAY—MCG. PER ML.

| Sample | Bioassay | Colorimetric | Fluorometric |
|---|---|---|---|
| Capsules | 830 | 900 | 898 |
| Capsules | 1110 | 770 | 770 |
| Capsules | 1150 | 820 | 836 |
| Troches | 139 | 140 | 154 |
| Troches | 149 | 137 | 150 |
| Troches | 140 | 140 | 154 |
| Troches | 110 | 147 | 154 |
| Capsules | 980 | 960 | 960 |
| Powder | 1260 | 1120 | 1100 |
| Capsules | 1030 | 870 | 870 |
| Powder | 1170 | 1055 | 1105 |

Of prime importance to this laboratory was the application of these methods to the assay of commercial preparations of aureomycin. Solutions of a number of commercial aureomycin powders, capsules, and troches were assayed by both of the above methods and by bioassay. The results are tabulated in Table VI.

It is evident from these data that the chemical measurements by both methods are directly related to the biological potency. The close agreement of the results obtained by the two methods, which are based upon such different mechanisms, provides mutual affirmation of the accuracy of each method. Studies are under way to determine the applicability of the fluorometric procedure to determinations of aureomycin in body fluids.

## SUMMARY

Two physicochemical methods for the assay of aureomycin are described: a fluorometric method with a lower limit of 0.25 mcg. per ml., and a colorimetric method, having a lower limit of 20 mcg. per ml. The results obtained by the two methods are in very close agreement, and are directly related to biological potency of the antibiotic.

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# Book Reviews

*Viral and Rickettsial Infections of Man.* Edited by THOMAS M. RIVERS. The J. B. Lippincott Company, Philadelphia, 1948. xvi + 587 pp. 17 x 25 cm. 77 illus. Price $5.

Ten or twenty years ago this book could not have been written, for knowledge concerning virus and rickettsial disease was changing so rapidly that the book would have been completely out of date by the time it could be published. Although these fields are by no means static now, they are changing sufficiently slowly that the facts can be evaluated and fitted into broader patterns.

The first 125 pages of this book deal with methodology. Physical and chemical procedures, serological reactions, chick-embryo techniques, and the propagation of viruses and Rickettsia in tissue cultures are all well covered by such experts as W. M. Stanley, Max Lauffer, Joseph Smadel, E. W. Goodpasture, John Buddingh, and John Enders. In reading this portion of the book one is impressed by the tremendous contribution which improved methodology has made to progress in these fields.

In a section of nearly 20 pages, Dr. Kenneth F. Maxey discusses the epidemiology of these diseases. Again, because of the more or less illusive character of this class of organisms, their epidemiological characteristics present special problems.

The remaining chapters are devoted to specific viral and rickettsial diseases. Some of the diseases covered are viral encephalitis, rabies, poliomyelitis, infectious hepatitis, common cold, primary atypical pneumonia, influenza, smallpox, measles, mumps, yellow fever, dengue, the typhus fevers, the "spotted" fevers, Q fever, and scrub typhus. Each of these chapters is written by an expert and contains information on the history, clinical picture, pathological picture, diagnosis, treatment, epidemiology, and control measures for each disease where such information is available.

No small measure of the worth of this book resides in the careful and conscientious editing by Dr. Rivers. The price is so reasonable because of the subsidy by The National Foundation for Infantile Paralysis.

*Torchbearers of Chemistry.* By HENRY MONMOUTH SMITH. Academic Press, Inc., New York, 1949. 270 pp. 17.5 x 25.5 cm. Price $8.

*Torchbearers of Chemistry* is a collection of the portraits of the leading "torchbearers" in the important field of chemistry. The portraits were taken from the collection of the Chemistry Department of the Massachusetts Institute of Technology. Each portrait is on a separate page and a very brief biographical sketch is underneath. A useful feature is the bibliography of biographies compiled by Ralph E. Oesper. Those interested in the humanistic approach to chemistry will find this to be a fascinating book.

*Symposium on Nutrition of the Robert Gould Research Foundation. Volume I. Anemia.* Edited by ARTHUR LEJIWA. The Robert Gould Research Foundation, Inc., Cincinnati, Ohio, 1947. 194 pp. 15 x 22 cm.

For many centuries, anemia has been recognized as an important branch of pathology. During the past two decades, important strides have been made in distinguishing the many kinds of anemia and in developing their treatments. A symposium to discuss the many phases of this important subject could be expected to contribute much to offset the confusion which has arisen. The symposium reported in the pages of this book helped immeasurably in such a clarification. Here one will find the viewpoint of the biochemist, the nutritionist, the clinician, and the pathologist, as presented by such authorities as Maxwell M. Wintrobe, C. A. Elvehjm, William J. Darby, M. O. Schultze, Wolf W. Zuelzer, G. E. Cartwright, Carl V. Moore, George M. Guest, W. H. Sebrell, and Richard W. Vilter.

The broader implications of the anemic state are presented, after which consideration is given to the role of folic acid. Unfortunately the full realization of the presence of vitamin $B_{12}$ was not known at that time so that the picture of pernicious anemia is quite incomplete.

The role of the metabolism of iron and copper was well elucidated by Dr. Schultze, who has made major research contributions in that area. Other phases (clinical) were well covered by Drs. Moore, Cartwright, and Guest.

The anemias of vitamin B Complex and ascorbic acid deficiencies were discussed in a straightforward and fair manner.

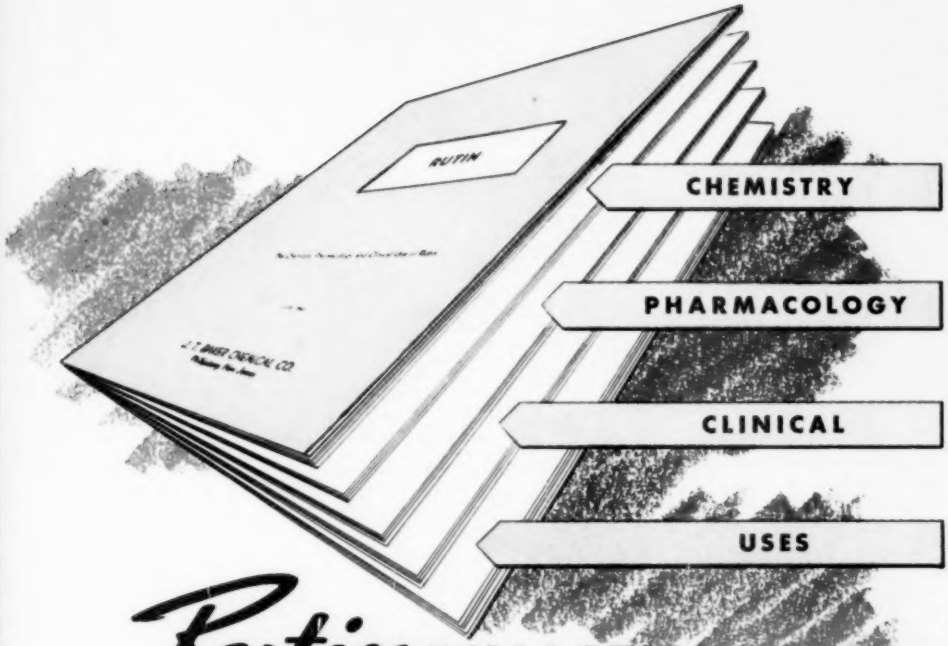
Symposia of this type are a welcome addition to the literature for they aid in separating the "wheat from a large quantity of chaff."

*Human Physiology.* By F. R. WINTON and L. E. BAYLIS. 3rd ed. The Blakiston Co., Philadelphia, 1948. xv + 592. 14 x 23.5 cm. Price $7.

Many in the field of pharmaceutical teaching will welcome the third edition of this excellent and standard text in physiology. The book owes its charm to the fact that it maintains the medical (and thus the pharmaceutical) point of view without becoming bogged down with the abundance of detail found so frequently in medical texts.

Although the text is British in origin, the authors have carefully reviewed the American literature and present a nice balance between Continental, British, and American physiology.

The text gives evidence throughout, of thorough revision and modernization. The documentation is selective and representative. The typography, binding, and paper stock are of the high quality usually used by the publisher.

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